Excipient Mediated Biostabilization of Protein Using Spray Drying Technique

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CERTIFICATE

This is to certify that thesis entitled, "**Excipient mediated biostabilization of protein using spray drying technique**" has been done under my guidance is a bona fide record of work done by Mrs. Priyadarsini Pattnayak in partial fulfillment of the requirement for the completion of the Master of Technology by research in Department of Biotechnology & Medical Engineering.

To the best of my knowledge, the matter embodied in this thesis has not been submitted to any other university/ institute for award of any Degree or Diploma.

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Abstract

Biologically active molecules like proteins are better preserved in dry condition owing to lower water activity and lower intra-molecular movement. However, 3-D conformation of protein is dependent on water around it. Upon drying, water around the protein is lost and therefore, excipients that work as water substitute molecule is provided during drying to preserve its 3-D structure. Bovine serum albumin (BSA) is taken as a model protein in this study. BSA is one of the highly studied proteins. Easy accessibility, low cost and inert behaivour enhances its significant utility in various biochemical formulations. In this study, BSA and excipient-BSA combinations were subjected to different levels of heat stress and the resulting structural changes were assessed using native gel electrophoresis, DSC (Differential Scanning Calorimetry), FTIR (Fourier Transform Infrared Spectroscopy) techniques. Native PAGE, FTIR, DSC techniques are used to determine the aggregation, secondary structure and glass transition properties of the spray dried and heat treated proteins respectively. The moisture content of the spray dried samples was determined by Thermo gravimetric analysis (TGA). For a protein to be stable, low moisture content, high Tg values are ideal. Protein aggregation has a direct link with its secondary structure. Thus, upon heat treatment of BSA and excipient-BSA combinations it is important that protein does not aggregate and the secondary structure remain intact for the protein to be stable.

Sugar, sugar alcohol and salt based excipients are well-known water substitute molecules. In the current study, BSA is spray dried in the presence of excipients like trehalose, mannitol, maltodextrin and ammonium sulfate in a ratio of protein:excipient, w/w 1:2. Further, the spray dried BSA samples were subjected to different levels of heat stresses and the resulting structural changes were monitored. Heat stress disrupts the 3-D structure of proteins by disturbing the secondary structure that ultimately leads towards protein aggregation.

Mannitol was found to be the best excipient in this study. Mannitol stabilizes the secondary structure of BSA. The α-helix at 1655 cm^{-1}, 3_{10} helix at 1665 cm^{-1} and β-turn at 1687 cm^{-1} of BSA remained intact for heat treatment at 65°C and 75°C and different time intervals (5, 10, 40 and 60 minutes) as monitored by IR spectroscopy. The recorded glass transition temperature (T_g) is quite high for Mannitol-BSA combination i.e. 75.1°C as quantified by

DSC. After a prolonged heat treatment for 60 minutes at 75°C the protein did not aggregate as clearly exhibited by native PAGE. Besides, the moisture content of the spray dried mannitol-BSA combination remains lowest i.e. 3.1 %.

Ammonium sulfate offers stability to the helical portion for short time at 65ºC and 75ºC up to 10 minutes of heat treatment**.** The structural change in case of ammonium sulfate-BSA combination monitored by FTIR is comparable to its native PAGE analysis. Long term heat treatment at high temperature caused aggregation in ammonium sulfate-BSA combination. BSA dried with trehalose and maltodextrin (Trehalose-BSA and Maltodextrin-BSA combinations) were found to be sticky, high moisture content and aggregated even during the spray drying. Consequently, they had low Tg, aggregates at low heat treatment and its secondary structure get easily disturbed as monitored by DSC, PAGE and FTIR respectively. Thus a comprehensive study on "excipient mediated biostabilization" of a model protein like BSA was accomplished in this thesis.

Keywords: Spray drying, Bovine Serum Albumin, Aggregation, Native Gel Electrophoresis, Moisture content, Thermo gravimetric Analysis, Glass transition temperature, Differential scanning Calorimetry, Fourier Transform Infrared Spectroscopy

ABBREVIATIONS

BSA	Bovine serum albumin
FTIR	Fourier Transform Infrared Spectroscopy
DSC	Differential Scanning Calorimetry
TGA	Thermo Gravimetric Analysis
PAGE	Poly Acryl amide Gel Electrophoresis
CD	Circular Dichroism
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
UV	Ultraviolet and Visible Spectroscopy
Tg	Glass Transition Temperature
Tc	Crystallization Temperature
Tm	Melting Temperature
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
Da	Dalton
SDS	Sodium dodecyl Sulfate
AS	Ammonium sulfate
DM	D-Mannitol
MD	Maltodextrin
T	Trehalose
Trp	Tryptophan
Phe	Phenylalanine
Try	Tyrosine
KBr	Potassium Bromide
TEMED	Tetramethylethlylenediamine
HCl	Hydrochloric Acid
NaOH	Sodium hydroxide
mM	Milimolar
Temp	Temperature

Chapter 1

Introduction

Biological systems are highly susceptible towards changes in their micro-environments**.** A small variation in the surrounding can affect the stability of the macromolecules, cells, intact plant and animal tissues. The factors that are liable to change the surroundings include temperature, pH, concentration, denaturants, etc. Bio-stabilization is a process for preserving and enhancing the shelf life of each and every component of the living systems individually and collectively. Besides being the vital component of the living systems, proteins are actively utilized in pathways like catalysis of biochemical reactions, transport of nutrients, recognition, and transmission of signals. Delicate structure of protein is stabilized by hydrogen bonds, hydrophobic interactions, disulphide bridges, van der waal forces etc. Any variations in the storage parameters can rupture the bonds, thus disrupting the dynamic structure and functions of protein. Proteins are subjected to various stresses during production, storage, and shipping, which increase the molecular motion and disturb the conformation. Helical structure of the protein i.e. α-helix and 3_{10} helix are energetically less stable and very sensitive to heat stresses. It can be disrupted and easily split leading to a change in 3D structure due to rupture of the hydrogen bonds. After breakage of the hydrogen bonds, protein may form aggregates. In the absence of a favorable environment, proteins are relatively unstable. Proteins are functional in their hydrated form. On prolonged storage in hydrated state, it loses its activity due to aggregation, disulphide exchange, oxidation etc. Thus proteins are prepared in dried form to enhance its shelf life. In the solid state, due to the removal of water protein loses its activity. Thus excipients forms hydrogen bonds with the protein molecule, create a water-like environment around the protein and strengthen the existence of its 3-D structure. The excipients protect its structure by providing the aqueous active surrounding even in the desiccated state. In order to clearly discriminate between a native and a denatured protein it is essential to have the understanding of denaturation of protein.

1.1 Denaturation of proteins

Alteration in secondary and tertiary structure may lead to denaturation if its 3D conformation is disrupted. The reason may be chemical, physical or thermodynamic one. In case of chemical degradation, protein may undergo deamidation, oxidation and aggregation. In deamidation process, the amide groups are removed by organic compounds. This is a pH dependant process which hydrolyzes asparagines to aspartate. Deamidation reactions can be checked by the addition of organic solvents (Yanli, 2000). The probability of oxidative damage in protein is very high. There are many residues like- Met, Cys, Trp, Tyr that can participate in oxidation reaction (Fransson et al., 1996). Disulphide bonds can play a major role in protein stability. Some times thiolate group of Cys residue pairs with the sulfur atom of a disulphide linkage that leads to breakage of the existing S-S bond in the polypeptide, creating a new link. This Disulphide exchange causes a structural change in proteins (Yanli, 2000). In order to balance interaction between hydrogen bonds and hydrophobic interactions which are disturbed by temperature and vortexing, some uncontrolled folding occurs, that is called as aggregation of proteins. Physical factors that responsible for denaturation are change in pH, concentration, and purity of proteins. Protein in stable form maintains a balance between folded and unfolded stage. The temperature has a major effect on the stability of the protein as it directly related to breaking of the secondary structure. With increase in the temperature the weak hydrogen bonds are disrupted and hydrophobic residues come out which leads to change in conformation (Jaenicke et al., 1990). Deviation from optimal pH conditions (low or high pH) results in hydrolysis and deamidation of proteins (Son et al., 1995). The Cys disulphide exchange reaction is influenced by pH and it also affects the rate of aggregation. Protein purity depends on the source and it is directly related to the protein stability (Brange et al., 1992). The presence of trace amount of metals or any other contaminant may affect the protein stability. Protein concentration also affects the aggregation formation, chemical degradation and disulfide bonding (Brange et al., 1992). Protein stability means avoiding the path of denaturation. There are many ways one can achieve it.

1.2 Protein Stabilization methods

Protein stabilization is highly desirable for storing the protein in its native form (Ragoonanan et al., 2007). Stabilization of structure requires preservation during and after processing. During production and storage, protein undergoes several biochemical changes that may lead to aggregation following various reactions. Protein conformation and stability is strongly held together by creating a favorable environment that will not allow the breakage of the intra-molecular interactions namely hydrogen bonds, hydrophobic and electrostatic interactions and disulfide bridges, etc.

1.2.1 Stabilizing the hydrogen bonds, hydrophobic and electrostatic interactions

As protein structure is balanced by various interactions, its stability can be enhanced by improving these interactions. It is reported that hydrophobic interactions contribute major part in the protein stability. Improving the hydrophobic interactions at interior and at the surface of protein can lead to its stability (Pace et al., 1992). Hydrogen bonds are very important for the stability of the secondary structure of the proteins. It is actively involved in the formation and maintenance of secondary structural components like α-helix, 3_{10} helix, β-sheets and turns. Hydrogen bonds are energetically very less i.e. 1.1–1.6 kcal/mol and break easily. Protein stability increases by increasing the strength of hydrogen bonds. The three dimensional structure of protein is strongly related to the salt bridge. The salt bridges can be improved by electrostatic interactions (Scholtz et.al., 1993). The strength of salt bridges offers a great impact on the stability of the proteins (Horovitz et al., 1990).

1.2.2 Lyophilization or Freeze drying

Proteins are generally stored in dry condition in order to avoid unnecessary interactions with bound water molecules and minimize water activity (A_w). Freeze drying is based on mechanism of dehydration. It is generally used for the preservation of heat sensitive material like protein. It works by freezing the material through mechanical refrigeration, dry ice and methanol, or liquid nitrogen and adding enough heat to allow the frozen water in the material to sublime directly from the solid phase to the gas phase. Lastly in secondary drying step, the unfrozen water molecules are removed. Through freeze drying, dry form of proteins are

produced with minimal damage to them and can be stored for years. Study of chymopapain stabilization by lyophilization was done (Philippe et al., 1997)

1.2.3 Structural modification through protein engineering and ligand binding

By improving the ligand binding site of a protein, the substrate interactions increases which increases the stability. The mechanism of the formation of complex is related to the thermal stability of the protein (Denisio et al., 1992). In general, introduction of disulphide bonds in between the residues using protein engineering tools improve the protein stability. Structural modification through protein engineering i.e. insertion or deletion of amino acids improve the stability of the protein and protect it from various stresses. Introducing a disulphide bond in between two identical subunits of Streptomyces subtilisin inhibitor increases the stabilization by increasing the melting temperature. Protein can also be stabilized by destabilizing the unfolding state as well as stabilizing the folding state. Intermolecular complex formation affects the stability of proteins (Denisov et al., 1992). The role of chaperon on protein folding was studied (Hartl et al., 1996). The various stabilization methods for protein stabilization was studied (Lee et al., 1997)

1.2.4 Immobilization

Structure can be modified by immobilization techniques. Immobilization can provide a noticeable protection from pH and high temperature (Michele et al., 1992). Stability of yeast alcohol dehydrogenase can be improved by covalent immobilization with glyoxyl-agarose. The half life of a protein can also be improved by modification with polymers (Jafri et al., 1993).

1.2.5 Spray drying

This process is based on evaporation of solvent mostly water. In spray drying the solution, suspension or emulsion is sprayed through a hot dry air. The liquid droplet dries within a fraction of second by the process of atomization which is carried out through a nozzle. This transformation of liquid to solid requires few seconds. Spray drying can be applied to many fields for creating amorphous solid with a specific particle size.

Spray-drying of therapeutic proteins has been successfully developed for the production of inhalable powders. Spray-drying an aqueous solution of a pure protein produces aggregation and/or loss of activity. These instabilities can, however, be ameliorated, in some cases fully,

by formulation measures. Disaccharides or surfactants in the liquid feed can prevent protein aggregation or inactivation during spray-drying (Broadhead et al., 1994). Spray-dried protein powders also have a potential application as a bulk storage material. If purified protein is insufficiently stable in aqueous solution, it is routinely freeze-dried to yield a storage stable powder. The most obvious disadvantage of this practice is the complexity of freeze-drying process control, which adds further cost to an already expensive process and cannot prevent occasional batch failure. Spray-dried protein powders represent, therefore, a potential alternative to such lyophilized bulk protein. To be suitable as a bulk storage material, a spray-dried protein powder must have a particle size sufficiently large to ensure the flowability necessary for bulk powder handling. A further major process requirement is a high powder yield from the spray-dryer. The remaining powder properties are, however, the same as those required for inhalable powders, viz. minimal protein damage during spray-drying, acceptably low residual moisture content, an overall spherical morphology, and satisfactory storage stability.

1.3 Improving the stability of the protein by addition of excipient

Excipient mediated protein stabilization has been widely studied. An excipient is an inactive substance used as a carrier for the highly active and thermo-labile biological and pharmacological substances. Pharmaceutical excipients are now designed in such a way that they should satisfy several essential qualities. It should bring multi-functional benefits to tablets and capsule formulations through great binding capability, improved dissolution properties, enhanced flow and lubricity. It offers benefit to product in terms of cost, productivity, and moisture protection.

Table-1.1: List of excipients used in various biochemical and biopharmaceutical industries.

Excipients	
Acetyltriethyl Citrate	Lactose and Cellulose
Aspartame	Lactose and Microcrystalline Cellulose
Alginates	Lauryl Lactate
Calcium Carbonate	Magnesium Stearate
Cellulose Acetate Phthalate-based Coatings	Maltodextrin
Cellulose-based Coatings	Poly ethylene Glycol
Cellulose and Lactose Combinations	Mannitol
Colorants for Film Coating Systems	Methylcellulose-based Coatings
Crospovidone	Microcrystalline Cellulose
Dextrose	Molasses
Dibutyl Sebacate	Shellac
Ethylcellulose-based Coatings	Sorbitol, crystalline
Fructose	Starch
Gellan Gum	Sucrose
Glycerol	Xanthan Gum
Lactose, anhydrous	Xylitol
Lactose, monohydrate	Trehalose

1.3.1 Excipients used in the current study

1.3.1 (a) *Trehalose*

The ubiquitous, non reducing trehalose is a natural alpha-linked disaccharide formed by α, α-1, 1-glucoside bond between two α-glucose units. The abundant sugar is synthesized as a stress responsive factor during various stress conditions. During dehydration of cell it forms a gel which prevents the organelles from disruption.

Table-1.2: Physical properties of Trehalose

Molecular Formula	$C_{12}H_{22}O_{11}$ (anhydride)
Molar mass	342.296 g/mol (anhydrous) 378.33 g/mol (dihydrate)
Appearance	White, Crystalline, odorless
Density	1.58 g/cm^3 at 24°C
Solubility	Soluble in ethanol, insoluble in diethyl ether and benzen

Significant uses of Trehalose

Trehalose is used in many food and pharmaceutical industry as a

- Food ingredient
- Protectant from drying
- Monoclonal antibody formulations
- Preservative for tissue and protein

Trehalose as an excipient:

Trehalose can be used as an excipient for the stabilization of proteins, animals, plants and microbial cells during drying. It gives a very high T_g value as detected by calorimetry. It is inert in several biochemical reactions and its low hygroscopic property makes it an excellent excipient in pharmaceutical and other industries. The stability of spray dried product IgG_1 was found to be improved by addition of trehalose. In the case of cutinase, it is found that trehalose formulation delays its thermal unfolding (Baptista et al., 2008). Trehalose protects the myoglobin by preserving its internal mobility. Trehalose form completely amorphous products and also stabilizes recombinant HSA for several months at 35°C determined by electrophoresis. This stress responsive factors has also proved useful in the cryopreservation of sperm and stem cells.

1.3.1 (b) ***Maltodextrin***

Maltodextrin is a polysaccharide composed of long chains of D-glucose units linked together by α (1→4) glycosidic bond. Partial hydrolysis of starch produces Maltodextrin. It is available as a white powder, sweet and flavorless.

Table-1.3: Physical property of Maltodextrin

Molecular formula	$C_{6n}H_{(10n+2)}O_{(5n+1)}$
Molar mass	Varies
Appearance	White powder

Significant uses of Maltodextrin

- Production of natural sodas and candy
- food additive
- Use as an excipient
- Use in healthy drinks, for being easily digestible and to increase the amount of calories. It can be used as a carbohydrate component in dry beverage mixes, puddings, desserts, cake, cookie, infant foods, ice creams, food coating, low alcohol beer, cheese spread and salad dressings, meal replacement powder, weight gain powder and post exercise supplements.

Maltodextrin as an excipient

Maltodextrin along with sucrose was able to protect the native structure of catalase. It can be used as a pharmaceutical excipient (Nath et al., 1992). Maltodextrin was used as a lyoprotectant for Lactate dehydrogenase. It gave better protection to the enzyme during freeze drying than sucrose and maltose as measured by the activity assays and calorimetry (Sun et al., 1998).

1.3.1 (c) Ammonium sulfate

Ammonium sulfate contains 21% nitrogen as ammonium cations, and 24% sulfur as sulfate anions. It can act as a soil fertilizer.

Table-1.4: Physical properties of Ammonium Sulfate

Molecular Formula	$(NH_4)_2SO_4$
Molar mass	132.14 g/mol
Appearance	Fine white hygroscopic granules or crystals
Relative Density	1.769 g/cm^3 (20 °C)
Solubility in water	70.6 g/100 ml (0 °C)
Solubility in other solvents	insoluble in acetone, alcohol and ether

Significant uses of Ammonium sulfate

- Preparation of other ammonium salts
- A food additive
- Purifying proteins by precipitation
- Artificial fertilizer for alkaline soils

Ammonium Sulfate as an excipient

Ammonium sulfate is used to stabilize human and horse serum butyrylcholinesterase at a few site. Ammonium sulfate was also able to give protection to the model enzyme lactate dehydrogenase against heat and urea mediated stresses.

1.3.1 (d) *Mannitol*

Mannitol is a sugar alcohol. It is a non reducing acyclic sugar, available in α,β, and γ forms

Table-1.5: Physical properties of Mannitol

Molecular Formula	$C_6H_{14}O_6$
Molar mass	182.172 g/mol
Appearance	Fine white hygroscopic granules
Solubility in water	Soluble in water

Significant uses of Mannitol

- To treat the patients with oliguric renal failure.
- Circuit prime of a heart lung machine during cardiopulmonary bypass
- Reduce acutely raised intracranial pressure

Mannitol as an excipient

The pharmaceutical protein recombinant humanized anti-IgE monoclonal antibody (rhuMAbE25) was spray dried with mannitol produced stable products (Constantine et al., 1998). Sugar alcohols and metabolites such as proline and glycine is said to protect diverse range of organisms against environmental stress. The protein and mannitol under vaccum drying was investigated (Sharma et al. 2004).

1.4 Problem Outline and Objectives

Excipient mediated bio-stabilization of proteins involve selected excipients, a model protein, a dehydration (drying) methodology and several analytical tools to study the stability aspects of the protein. Since the goal is to evaluate the excipients that are capable of stabilizing the protein for longer time at room temperature conditions, accelerated degradation of proteins are generally adopted to evaluate and characterize the effect of excipients on the protein's structure and activity during extreme conditions. Heat stresses caused by high temperature heat treatment, acidic and/or alkaline environments, elevated humidity and photon bombardment expedite the degradation process of proteins. Native proteins in aqueous environment crumble quickly in either one or combination of such stresses. Proteins that are dehydrated in the presence of excipients are very well immobilized, have less bound water and less water activity (A_w), placed in a glassy matrix of excipients that have high glass

transition (T_g) temperature keeping the protein intact in immobilized state. Thus, excipient-protein combinations resist the accelerated degradation. It is to be seen that to what extent a particular excipient resist the degradation of the protein. In this study, protein:excipent, w/w 1:2 combinations are first of all dehydrated using spray drying technique and heat stress is applied to accelerate the degradation of immobilized protein. Finally, the effects of heat stress on the protein-excipient combinations were evaluated by PAGE and IR techniques.

Keeping the above mentioned overall goal in mind we set the following objectives:

1. To spray dry one excipient at a time in combination with BSA in a ratio of protein:excipient, 1:2 w/w.
2. To subject the native protein and other spray dried protein samples to a known heat stress (65 $^{\circ}$C and 75 $^{\circ}$C) for a known time period (5, 10, 40 and 60 mins)
3. To quantify the moisture content and glass transition temperature of spray dried protein-excipient formulations using TGA and DSC techniques
4. To analyze the structural changes in the protein exposed to accelerated degradation using native PAGE and FTIR techniques

Chapter 2

LITERATURE SURVEY

2.1 Bovine Serum Albumin

Bovine serum Albumin accounts for about 60% of blood plasma protein, hence regarded as the most abundant protein content of blood. In mammals, albumin is synthesized as preproalbumin by the liver. The albumin released into circulation possesses a half-life of 19 days (Carter et al., 1994).

2.1.1 Structure of Bovine serum Albumin

The widely studied Bovine serum albumin (BSA) possesses molecular weight of 66500Da (Pico et al., 1996). Albumin from different mammalian species show many similarities in physico-chemical criteria. The indistinguishable bovine (BSA) and human (HSA) serum albumin shows nearly 76% of sequence identities, similar surface hydrophobicity and also in partitioning behavior (Michnik et al. 2006). This globular protein contains three domains, each domain formed by 6 helices, and 9 loops stabilized together by internal networking of disulphide bonds. Each domain contains two sub domains. Bovine serum albumin contains 583 amino acids containing two tryptophan (Trp-134), located in Proximity of the protein surface, but buried in a residues located in the domain-I and Trp-236 located in the internal part of the domain-II (Ajloo et al., 2006). Its primary structure is characterized by low content of tryptophan, a high content of cystein which stabilizes a series of main loops, and charged amino acids such as aspartic acid, glutamic acid, lysine and arginine. Its secondary structure contain 67% of α-helix, 10% turn, 23% extended chain, and no β-sheets. From FTIR analysis, it was proposed that BSA does not denature up to 40 °C. Conformational changes of the BSA molecule are reversible in the temperature range of 42- 50 °C, but unfolding of α-helices of BSA is irreversible in the temperature range of 52-60 °C. From 60 °C, the unfolding of BSA progresses and β-aggregation of the BSA molecule begins. Above 70 °C, the gel formation by unfolding of BSA advances further (Murayama et al., 2004)

2.1.2 Applications in diverse fields

BSA is one of the most extensively used proteins due its availability, low cost, stability, and unusual ligand binding properties (Curry et al., 1998). In restriction digests, BSA is used to stabilize some enzymes during digestion of DNA and to prevent adhesion of the enzyme to reaction tubes and other vessels. BSA is also commonly used to prepare a protein stabdard plot that is needed for estimation of other proteins. In addition, it has been reported that albumin is chiefly responsible for maintenance of blood pH (Carter et al.1994). The serum albumin is a multifunctional plasma carrier protein because of its unusual ability to bind with a broad spectrum of ligands which include inorganic cations, organic anions, various drugs, amino acids, and perhaps most important and physiologically available hydrophobic molecules such as bilirubin and fatty acids. Serum albumin is regarded as drug carrier in the drug delivery system. It interacts with the drugs and helps in drug design (Honda et al., 1999). Though albumin is a protein, it can also function like an enzyme. There are various applications of albumin in clinical areas like: it can be used as a fatty acid carrier for biosynthesis of lens lipids. BSA with fatty acid showed the highest breaking strength and evaluated as having highest wielding ability.

2.1.3 Structural changes occurs due to heat stress

BSA has a tendency to aggregate in macromolecular assemblies. As temperature increases, the hydrogen bonds become weak and they break. Some molecular regions become accessible to new intermolecular interactions, producing soluble aggregates through disulphide and non-covalent bonds (Yanli , 2000). Specifically, BSA aggregation appears to be the result of three interconnected mechanisms: critically diverging concentration fluctuations, conformational changes and proteins cross-linking. The degree to which it happens is temperature and time-dependent. Moreover, intermolecular β-sheet formation can take place and contribute to intermolecular association of the partially unfolded protein molecules. All these intermolecular interactions can give rise to the protein gelling phenomenon and is driven by surface net charge and hydrophobic area exposed to the solvent. Serum albumin when heat-treated, goes through two stages (Kuznetsow et al., 1975). The first stage is reversible and is carried out at 65°C while the second stage is irreversible which requires a high temperature but does not necessarily result in a complete destruction of the ordered structure.

2.1.4 Previous work done

The conformational changes of BSA due to thermal aggregation was studied by using steady state fluorescence and circular dichroism. The two tryptophan residues found in different domain of the protein were targeted in order to study the mechanism of change by using fluorescein-5-maleimide as dye. Differential scanning calorimetry was employed to study the thermal denaturation processes of the main protein fractions of blood serum like α,β-globulins, γ-globulins, human serum albumin with or without fatty acids. By changing the concentration protein stability can be studied. In order to detect the relationship between the concentration and protein stability was studied by using various the concentrations of BSA. Aggregation of BSA due to thermal stress was determined by native gel electrophoresis (Arakawa et al., 1999). Thermostability of BSA and HSA was checked by DSC (Michnik et al., 2006). The changes that occurred in the structure of the BSA due to temperature 130°C were studied (Moriyama, 2008).

2.2 Conventional techniques for protein structure determination and their limitations

Table-2.1: Different techniques used for the detection of structure of proteins

Techniques	**Information about the structure of protein**
Mass Spectrometry	Primary Structure
Fourier Transform Infrared Spectroscopy (FTIR)	Secondary Structures
Raman Spectroscopy	Secondary Structures
Circular Dichroism Spectroscopy (CD)	Far UV-Secondary Structure Near UV- Tertiary structure
Ultraviolet/Visible Spectroscopy (UV/VIS)	Tertiary Structures
Fluorescence Spectroscopy	Tertiary Structures
Nuclear Magnetic Resonance Spectroscopy (NMR)	Tertiary and Quaternary Structures

X-Ray Crystallography	Tertiary and Quaternary Structures
Electrophoresis	Aggregation or Fragmentation and also tertiary structure in some cases
Differential Scanning Calorimetry (DSC)	Thermal Stability
Thermo gravimetric Analysis (TGA)	Thermal Stability

2.2.1: Mass spectroscopy

It is an analytical technique used to determine the elemental composition, chemical structure of a sample by ionizing them to generate charged molecules or molecular fragments

Limitations

- Restricted to some proteins based on their molecular weight
- Able to give information about protein primary structure only

2.2.2: Raman spectroscopy

Raman scattering is a powerful spectroscopic technique is based on the principle that light of a known frequency and polarization is scattered from a sample. The scattered light is used to diagnose the internal structure of molecules and crystals. Raman spectroscopy can be applied for reaction monitoring, chromatograpic detection, environmental monitoring and corrosion studies. Raman spectroscopic study on different protein like BSA was done in order to detect the sequence and primary structure (Izutsu et al. 2002).

Limitations

- Detection through Raman spectroscopy many times not possible because it produces weak signals
- High rejection of scattered laser light must be provided by the spectrometer

2.2.3: Circular Dichroism

This spectroscopic study is very simple and is regarded as the extensively sensitive to protein secondary structure. It can give information both in qualitative as well as qualitative manner. Optically active chromophores absorb different amount of right and left polarized light, this difference in absorbance results in either a positive or negative absorption spectrum. A weaker CD spectrum is obtained along with an increasing degree of denaturation. Even if there are high concentrations of denaturants the protein conformational stability can still be monitored by CD spectra.

Limitations

- Extremely sensitive to local environment and the conformational changes near the aromatic groups and the disulfide bonds (Prieto et al., 1997).
- Hard to quantitate the secondary structural component percentages.

2.2.4: Ultra violet spectroscopy

The optical spectroscopic technique spectroscopy is one of the most important methods to determine protein properties. It is a reliable accurate analytical laboratory assessment procedure for analysis. Targeting chromophoric groups it can provide information about protein concentrations and the immediate environments. The change in UV or fluorescence signal can be negative or positive, depending on the protein sequence and solution properties.

Limitations

- Absorbance cannot accurately indicate either conformational stability or protein concentration

2.2.5: Fluorescence Spectroscopy

The dynamic and noninvasive fluorescence measures the emission energy after the molecule has been irradiated into an excited state. Since the fluorescence is related to the solvent conditions even in the absence of conformational changes, it is useful to study solvent-protein interactions. Protein stability was monitored by fluorescence spectroscopy (Eftink et al., 1994)

Limitations

- Very sensitive and by quenching process fluorescence intensity of a given substance decreases and accurate results can not be obtained (Davis et al., 1987).

2.2.6: Nuclear Magnetic Resonance Spectroscopy

NMR is the foremost experimental method to study dynamic properties of proteins, nucleic acids, a variety of low molecular weight compounds of biological, pharmacological and medical interests. NMR can generate structures at atomic resolution. It can be applied in solution with an adequate amount of proteins (Middaugh et al., 1990). At the atomic resolution, 3D structures and secondary structure of proteins can also be determined (Xu et al., 1997). It can provide estimation for both of the extent and location of the secondary structure (Reid et al., 1997). Kinetic study of protein was done by NMR method (Dobson et al., 1998)

Limitations

- Complex interpretation
- Can be applied to small proteins and multidimensional NMR may allow a molecular weight up to 60 kD (Clore et al., 1998).

2.2.7: X-ray crystallography

X-ray crystallography is regarded as the most important and investigational technique that applies the X-rays to prepare crystals structures. It can reliably provide 3-D molecular structures, from global folds to atomic details of bonding. The knowledge of accurate molecular structures from X-ray is a prerequisite for rational drug design and for structure based functional studies, in order to develop the effective therapeutic drugs

Limitations

- Crystal formation is not available for most of the proteins.

- It can give only solid state information.

2.3 Techniques used in the study

2.3.1 Fourier Transform Infrared Spectroscopy

FTIR is flexible and used to give information in diverse range of environment. This powerful laser detects the denaturation of proteins by analyzing the secondary structural components which are generally α-helix, β-sheets, β-turns and 3_{10} helix. When a sample is placed in IR source, the energetic light creates molecular vibrations at a particular wavelength. IR spectroscopy monitors the vibrational intensity i.e. occurs due to stretching or bending and wave length of absorption by a sample. Total 9 characteristics of Amide bands are present in the peptide sequences of protein. Amide-I and Amide-II are associated with the IR spectrum. Amide-I arises due to C=O stretching and usually related to the backbone conformation. It is generally located in the region of 1600 cm^{-1} to 1700 cm^{-1} (Kong et al., 2007).

Table-2.2: The characteristic IR bands of the proteins and peptides

Designation	Approximate frequency (cm^{-1})	Description
Amide-A	3300	NH stretching
Amide-B	3100	NH stretching
Amide-I	1600-1690	C=O stretching
Amide-II	1480-1575	CN stretching, NH bending
Amide-III	1229-1301	CN stretching, NH bending
Amide-IV	625-767	OCN bending
Amide-V	640-800	Out-of-plane NH bending
Amide-VI	537-606	Out-of-plane C=O bending
Amide-VII	200	Skeletal torsion

The above data obtained from Elliott and Ambrose, Krimm and Bandekar, Banker and Miyazawa et al.

2.3.1 (a) Band Assignments

Amide-II band is found in between 1510-1580 cm^{-1}. It arises due to N-H bending, C-N and C-C stretching. α-helix is found at the region of 1548 cm^{-1} in Amide-II. In Amide-I,

β-sheet absorption is nearly 1640 cm^{-1} and α-helix absorbs near 1652 cm^{-1}. Amide-I is more sensitive than the Amide-II. By analyzing the peaks at corresponding region, the structure and the changes can be marked.

For analysis, there are many methods have been used like-curving fitting method usually meant to quantitate the area of each component. BOMEN GRAM/32 software can be used to estimate data analysis. There are several softwares are used to quantitate the relative area of each component. Data analysis can be done by taking the double derivative of the spectrum.

Table-2.3: Deconvoluted amide I band frequencies and related secondary structure structural components of protein in H_2O media.

Mean frequencies (Hz)	**Assignment**
1624±1.0	β-sheet
1627±2.0	β-sheet
1633±2.0	β-sheet
1638±2.0	β-sheet
1642±1.0	β-sheet
1648±2.0	Random
1656±2.0	α- Helix
1663±3.0	310 Helix
1667±1.0	β -Turn
1680±2.0	β-Turn
1685±2.0	β-Turn
1691±2.0	β-sheet
1696±2.0	β-sheet

2.3.1(b) Advantages of FTIR

- It can produce accurate results with less amount of samples
- Fast and non invasive
- Gives information at any type of environment and easy to operate
- Gives information about secondary structure, stability and also structural stability of proteins

The only limitation is that it requires different softwares for data analysis

2.3.1 (c) Structural changes of proteins detected by FTIR

FTIR is a well established analytical tool for the detection of protein structure. Pressure induced alterations in secondary structure of ribonuclease-A was studied by Conventional FTIR spectroscopic method. This spectroscopic technique has been carried out to investigate the thermal denaturation of α-actinin (Han et al. 1998). Sugar additives like sucrose, trehalose improves the stability of Human serum Albumin during Lyophilization. Infra red method can be used to study hemoglobin reactions (Dong et al., 1994)

2.3.2 Electrophoresis Methods to Study Aggregation or Fragmentation

Electrophoresis is an analytical technique used to separate and purify macromolecules like proteins and nucleic acids based on their size, charge or conformation. For the separation of proteins, PAGE (Poly Acrylamide Gel Electrophoresis) is used. PAGE is basically of two types: Native PAGE and SDS-PAGE.

2.3.2 (a) Native Gel Electrophoresis

Native or non-denaturing gel electrophoresis is run in the absence of any denaturant like SDS and used for separation of non denatured proteins. This process gives clear cut definition about the protein native structure.

Electrophoresis separation in a native gel may produce information both on sizes and charges. The disadvantage is that it is a time taking

The non denaturing gel electrophoresis is used to study the kinetic and thermodynamic properties of human erythropoietin (Arakawa et al. 2000).

2.3.2 (b) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

It is very common process which gives the information about the molecular weight of an unknown protein. It can not give any information about the native form of proteins.

2.3.3 Differential scanning calorimetry

It is a thermo analytical process by which the physical changes of a substance can easily be detected. As the enthalpy change, is a function of temperature of the sample increased or decreased, it is measured by DSC. The specific heat of a sample can be calculated by measuring the energy required to increase in the temperature of the sample. As the sample is provided through a constant range of temperature the physical transitions occurred and these are measured through DSC. This transitions occurred due to making and breaking of the bonds there by enthalpy transitions can be determined. The effect of surfactant like SDS on the stability of BSA was studied and detected by DSC (Giancola et al. 1997)

2.3.3 (a) Principle of operation

When a constant amount of heat is supplied to a sample it undergoes state change phenomena. During physical change, sample forms different transitions. The transitions include glass transition (T_g), crystallization (T_c), melting (T_m). Based on these thermal transitions, protein stability can be detected. It can be use to characterize thermal properties of materials and determine the heat capacities, melting enthalpies and transition points accordingly by rate. Depending on the varying thermal behavior of the sample, the energy will be taken or diffused from the sample, and the temperature difference will be sensed as an electrical signal to the computer. As a result, an automatic adjustment of the heaters makes the temperature of the sample holder identical to the reference holder. DSC can also be used to study the water of hydration of pharmaceuticals and excipients. DSC can be applied to the thermal analysis of impurity of raw materials during in-process, scale-up and quality control. It can also be used to determine the storage conditions of finished products. It is very sensitive to concentration of the protein i.e. if the accurate concentration is not maintained then it will not produce the perfect result. DSC measured the stability of proteins by measuring the T_m value. The effect of different denaturants like SDS on BSA is detected by

Differential scanning Calorimetry. Themo sensitive enzyme like yeast alcohol dehydrogenase can be stabilized by sucrose detected by Calorimetry (Nath et al.1997).

2.3.3 (b) Glass transition temperature

It is a critical temperature at which the material undergoes a change in the physical state i.e. from hard, brittle state to a rubbery, elastic and flexible state. In glass, there are no particular molecular arrangements. In glassy state, the molecular motion is arrested due to high viscosity. Polymers in amorphous state depend on the mobility of the polymers and minimization of Gibb's free energy.

2.3.3(c) DSC in some other uses

- Protein folding, unfolding and stability study
- Biopharmaceutical formulations development and characterization
- Process development.
- Protein engineering studies
- Rank order binding.
- Antibody domain binding studies.
- Characterization of membranes, lipids, nucleic acids and micellar systems.
- Assessment of the effects of structural change on a molecule's stability.
- Assessment of biocompatibility during manufacturing.

2.3.4 Thermo gravimetric Analysis (TGA)

TGA measures sample mass loss and the rate of weight loss as a function of temperature or time. TGA provides an important tool to characterize and quantify the moisture content in pharmaceutical materials. The measurements are used primarily to determine the thermal and/or oxidative stabilities of materials as well as their compositional properties. The technique can analyze materials that exhibit either weight loss or gain due to decomposition, oxidation or loss of volatiles (such as moisture). The thermal analysis of protein is carried out to measure the weight loss of Lysozyme (Liao et al. 2002).

2.3.4 (a) Principle of operation

A test sample is placed in an alumina/platinum sample holder that is attached to an analytical balance. After that the sample is heated up to certain temperature maintained by a

thermocouple as it has to be set before. TGA curves shows percentage of weight verses temperature**.**

2.3.4 (b) TGA in some other uses

- Analysis of materials
- Decay temperatures
- Degradation rate
- Product self life
- Damage due to oxidative stress
- Evaluation of polymer flammabilities
- Thermal stabilities studies
- Identifying the polymers through Fingerprinting
- Moisture and volatile Content
- Analysis and characterize the evolved gases using TGA
- Effects of various parameters on materials

Moisture induced aggregation via disulfide scrambling was also detected in lyophilized proteins such as bovine serum albumin, ovalbumin, glucose oxidize, and β-lactoglobulin.

2.3.5 Spray drying

Spray drying is a one-step powder generating operation from liquid feed to its dried particulate form through exposure to drying air. Air is used as a heated drying medium, and it may flow along with the direction of the product and also in the opposite to the product. There are several constructive designs and approaches for spray dryer. A systematic approach was developed in order to get a solution form rational spray dryer that leads to save cost, time and easy to handle (Nath et al. 1998). The effect of excipient and spray drying parameters was studied (Andya et al. 1999).

2.3.5 (a) Instrumental Operation - Spray dryer takes the feed through a polypropylene pipe by a pump. Then the liquid steam comes in contact with the atmospheric air drawn through the air filter. There is a heater in the instrument in order to convert the atmospheric air to hot drying gas. The aspirator motor produces the required amount of vacuum in order to carry out the process The liquid steam is spray dried through the nozzle and products are collected

through the drying chamber and in the cyclone separator collection pot and the air is released to the atmosphere. These are various parts of the spray dryer.

- Aspirator
- Compressor
- Pump
- Heater
- Nozzle
- Drying chamber

2.3.5 (b) Applications in various fields-

Table-2.4: Spray drying applications

Pharmaceutical industry	Antibiotics, medical ingredients, Additives
Food industry	Milk powder, Coffee, Tea, Eggs, Cereal, spice, Flavorings
Industrial	Paint pigments, ceramic materials, catalyst supports, Bone and tooth amalgams Beverages, colorings and plant extracts Milk and egg products Plastics, polymers and resins Soaps and detergents Textiles etc

2.3.5 (c) Spray drying of proteins

Sucrose and trehalose preserve the lysozyme native structure when they are spray dried with the protein. The activity and stability of trypsinogen after spray drying was studied by (Tzannis et al., 1999). The effect of sucrose, trehalose, mannose, on the thermal stability of the protein transglutaminase by spray drying was studied (Cui et al., 2000). Trehalose protects yeast alcohol dehydrogenase from stress through spray drying (Yosii et al. 2007).

The widely used excipient trehalose protects the model protein lactate dehydrogenase through spray drying (Adler et al. 1999). The various effects of parameters and formulations on the preservation of spray dried β-galactosidase was examined (Broadhead et al. 1994). The change in shape and morphology of three spray-dried protein powders were analyzed by scanning electron microscopy, X-ray diffraction and residual moisture analysis (Costantino et al.1997). In order to study the adsorption behavior of proteins BSA and β-lactoglobulin were spray dried and results were analyzed by fluorescence quenching mechanism (Landstrom et al. 1999). The activity and stability of trypsinogen effect of sucrose during spray drying was studied (Tzannis et al. 1999).

Chapter 3

Materials and methods

3.1 Materials

The following chemicals are used in the study.

Table- 3.1: Different chemicals used in the experiments

Serial No.	Chemicals with Catalogue No.	Company Name
1	Bovine Serum Albumin (11201800101730)	Merck Private Limited
2	Trehalose (625625)	Calbiochem
3	Mannitol (134889)	Sisco Research Laboratories Private Limited
4	Ammonium sulfate (61750705001730)	Sisco Research Laboratories Private Limited
5	Maltodextrin (RM 1249)	Himedia laboratories Private Limited
6	Hydrochloric acid (HCl)	Himedia, laboratories Private Limited
7	sodium hydroxide (NaOH)	Himedia, laboratories private Limited
8	NaH_2PO_4 (61784505001730)	Merck Private Limited
9	Na_2HPO_4 (61754905001730)	Merck Private Limited

10	Acrylamide (8.008301000)	Merck Private Limited
11	Bis acrylamide (REF MB 005)	Merck Private Limited
12	Ammonium persulfate (RM1096)	Merck Private Limited
13	Bromophenol blue (REF RM 117),	Merck Private Limited
14	Coomassie brilliant blue-G (REF RM 1219),	Merck Private Limited
15	TEMED (MB026)	Merck Private Limited
16	Acetic acid (AS001)	Merck Private Limited
17	Glycerol (61756005001730)	Merck Private Limited
18	Methanol (82228305031730)	Merck Private Limited
19	Potassium bromide (P0220)	RANKEM, Ranbaxy

The required equipments used in study are given below.

Table- 3.2: Instrument used in the study

Serial No	Equipments	Source
1	Water bath	LAUDA Ecoline- staredition RE-104
2	Refrigerator	LG
3	Analytical balance	Afcoset ER-200A
4	Ultra pure water system	Millipore
5	Ultra Low Temperature freezer	Remi- RQFP 265
6	pH meter	Systronics
7	Vortex Mixer	Genie
8	Spray dryer	Labultima-LU20
9	Thermo gravimetric Analyzer	SHIMADZU, DTG-60H
10	Electrophoresis	Genie
11	Differential scanning calorimetry	Jupiter-NETCSCH STA 449C
12	Fourier Transform Infrared Spectroscopy	Perkins Elmer-Spectrum RXI

3.2 Methods

3.2.1 Spray drying of BSA with different excipients

Fig. 3.2.1 Laboratory Spray Dryer (Labultima-LU-20)

3.2.1 (a) Parameter optimization

- Spray drying parameter optimization was done by considering the inlet air temperature, out let air temperature, aspirator motor speed, pump flow rate and feed concentration.
- In order to get the accurate parameter for protein, spray dryer was run at the following conditions like inlet temperature starting from 90ºC to160 ºC, accordingly the outlet temperature from 50°C to 140°C aspirator motor speed 40% to 55%, pump flow rate 8 to12 and the feed concentration 0.5gm/10ml,1gm/10ml, 2gm/ml etc.

The optimized parameters were-

- *Inlet temperature- 130°C*
- *Outlet temperature- 90°C*
- *Feed pump speed- 45*
- *Aspirator speed- 10*
- *Feed concentration- sample: distilled water :: 7.5g :50ml*

3.2.1(b) Spray drying

- For spray drying, the ratio of protein: excipient was 1:2 (w/w) i.e. 2.5 gm of protein and 5 gm of excipient dissolved in 50 ml of water. That was used for the preparation of the feed solution.
- All the parameters were set and the instrument was start for run. The air was drawn form the atmosphere by aspirator. Air filter supplies the filtrate air to the instrument. The atmospheric air was converted to hot gas by heater
- Before drying the sample solution single run of water would be done for internal deblocking. The pump flow was started after the unit gets the desired inlet air temperature.
- Through pump, feed solution was taken to the system and by hot gas it atomized in to droplets through nozzle. As the process continues, the final powder was deposited in the drying chamber and cyclone separator.
- After the completion of the sample solution, the instrument was again set for cooling by reducing the inlet air temperature below 100 $^{\circ}$C.
- The final dried product was collected from drying chamber collection pot and cyclone separator collection pot and kept for further study in desiccators.

3.2.2 Sample preparation:

Samples were prepared by giving heat treatment in a water bath at different temperature (such as 65 $^{\circ}$C and 75 $^{\circ}$C) with different time interval like 5, 10, 40 & 60 minutes. After heat treatment, they were put in ice for 2-3 minutes. These samples were stored in desiccators for maintaining a moisture free environment. These prepared samples were taken for the IR spectroscopy, DSC directly and for the Native Gel Electrophoresis they were taken along with the Phosphate Buffer. Sodium phosphate buffer was prepared by Disodium hydrogen phosphate (NaH_2PO_4-Molecular weight-156.01) and Sodium dihydrogen phosphate (Na_2HPO_4- Molecular weight-141.96) and pH was adjusted to 7.

3.2.3 Thermo gravimetric Analysis for protein formulations

Thermo gravimetric analysis was done in order to detect the moisture content of the sample. Both formulated as well as the native protein was analyzed by putting them in sealed chamber and the temperature was set 150ºC through the thermocouple. That gives the information about the moisture content measurement due to weight loss. The percentage of moisture content in the sample is determined by the formula:

$$\%\ of\ moisture = \frac{initial\ weight - final\ weight}{initial\ weight} \times 100$$

3.2.4 Native Electrophoresis of samples

Electrophoresis procedure was followed according to standard protocol.10% of native polyacryalamide gel was prepared in order to study the denaturation process.

3.2.4 (a) Preparation of the stock solutions

- Acryl amide monomer stock was prepared by adding acryl amide 30 gm, bis acryl amide 0.8gm and making the volume up to 100ml with distilled water.
- By addition of tris buffer-18.66 gm with distilled water 75 ml. separating gel buffer was prepared After adjusting the pH 8.8 with dilute HCl the volume was made up to 100ml.
- Stacking gel buffer was prepared by adding 6.55 gm of tris buffer was to 75ml of distilled water and then the pH was set to 6.8. Final volume was made up to 100ml by DW.
- Bromophenol blue about 0.1 gm mixed with glycerol 2ml and stacking gel buffer 2.5 ml for the preparation of sample buffer.
- Tank or running buffer was prepared by tris buffer 0.5 gm and glycine 14.4 gm. The pH was adjusted to 8.3 and volume was adjusted to 1000ml.
- In order to prepare the staining solution, coomassie brilliant blue 2 gm, methanol 500ml, acetic acid 100 ml dissolved in 500 ml distilled water.
- Distaining solution was prepared by methanol 500 ml, acetic acid 100 ml and distilled water 500ml. Stock solutions like-acryl amide monomer, stacking gel buffer,

separating gel buffer, running buffer, sample buffer were prepared and kept in refrigerator.

- After preparation the stocks were kept in the refrigerator. Ammonium per sulfate was prepared freshly every time.

3.2.4 (b) Gel preparation and run

- Running gel and stacking gel were prepared fresh for each run.
- By addition of acrylamide monomer 1.7 ml, separating gel buffer 1.3 ml, distilled water 1.9 ml, APS 50µl and TEMED 10µl running gel was prepared.
- Stacking gel was prepared by adding acryl amide monomer 0.5 ml, stacking gel buffer 0.4 ml, distilled water 2.1 ml, APS 30µl and TEMED 10 µl.
- After preparation of the running gel, it was poured in between the glass cascades which were sealed tightly. After the casting of the running gel, stacking gel was poured in to that and comb was inserted in order to form the wells.
- 10µl of samples along with sample buffers were loaded in the wells. The migrated samples produce bands under the influence of constant amount of electric field.

3.2.4 (c) Staining and de staining of the gel

Gels were stained in the coomassie staining solution and kept for 2-3 hours and after that destaining solution was added in order to visualize the bands.

3.2.5 Fourier Transform spectroscopy

Fig. 3.2.2 FTIR (Perkins Elmer-Spectrum RXI)

For FTIR spectroscopy, solid sample were used. Samples were prepared as described before in the water bath giving different temperatures and at various time points. Fresh prepared samples were taken for study. 2-3 mg of solid samples were milled with potassium bromide in order to mix properly. Sample and KBr ratio should be 1:3.

3.2.5 (a) Pellet preparation and scanning

- Pellets were prepared by adding the sample and KBr.
- The prepared pellets were scanned through the whole range starting from 4000cm^{-1} to 400 cm^{-1}. The plots were done by taking wave number against absorbance in X and Y axis respectively.
- For analysis part, the peaks of Amide-I and Amide–II bands were considered which is found in between 1600 and 1700$cm^{-1.}$

3.2.6 Differential scanning Calorimetry

Fig. 3.2.3 DSC Jupiter-NETCSCH STA 449C

Solid sample i.e. both formulated and as well as native protein at room temperature were put in the sample pan in a closed chamber of the instrument. The scanning was done starting from room temperature 100°C at a rate of 5°C per minute. The difference in the temperature between sample and reference was plotted against the specific heat of the sample. The DSC graph would show physical changes of the sample due to heat by measuring T_g, T_c and T_m of the sample.

Chapter 4

Results and Discussion

Protein denaturation is modification in conformation not accompanied by rupture of peptide bonds. It ultimately leads to totally unfolded polypeptide structure. Protein denaturation can either be reversible or irreversible. It can be due to change in pH, addition of several denaturants, due to heat and so on. Denaturation and aggregation of proteins are commonly associated with the alterations in the populations of the 3_{10} helix, α-helix, β-sheet, and β-turns. The temperature has a major effect on the stability of the protein as it directly related to breaking of the secondary structure. With increase in the temperature the weak hydrogen bonds are disrupted and hydrophobic residues come out which leads to change in conformation (Jaenicke et al., 1990). Low and high pH results hydrolysis and deamidation reactions (Sun et al., 1998).

Fig.4.1.1 Spray dried protein- Mannitol

Fig.4.1.2 Spray dried protein-Ammonium sulfate

Fig.4.1.3 Spray dried protein- Maltodextrin

Fig.4.1.4 Spray dried protein- Trehalose

4.1 Moisture content determination through Thermo Gravimetric Analysis (TGA)

Proteins are more stable in solid state than that of in aqueous solution. In presence of water they aggregates or may also form disulphide links in between the Cys residues thus leading to a change in the conformation and it also undergo several biochemical reactions like oxidation and deamidation. Thus presence of moisture affects the stability of the protein in a negative manner.

Thermo gravimetric analysis method has been employed in this study to find the percentage moisture content of proteins. For a protein to be stable at optimum condition, the percentage of moisture content should be less (Town et al., 2000). There are several reactions that take place in the presence of water there by making the protein dynamically active resulting in the change in its conformation. In the presence of water, glass transition value reduces and phase change occurs. Protein molecules transits from a constrained, viscous and supersaturated state to a relaxed state. The hydrogen bond breaks as the temperature increases there by it brings the change in the structure of the proteins. Molecular mobility is also increased due to increase in the water content. From thermodynamic point of view, the structural alterations occur due to the imbalance in the mobility of the molecules which leads to the change in ΔG. In this way the native balanced state of protein becomes imbalanced (Ragoonanan et al. 2007). The residual moisture content inversely correlate to the stability. Lippert et al. found that hydroxyl group plays a major role for maintaining the stability of phosphofructokinase and lactate dehydrogenase. Excipient plays a major role in stabilizing the proteins by satisfying the required hydrogen bonds in order to maintain its native structure even at the absence of its hydrated environment, during the deleterious reactions like phase separation and solute crystallization. They act as the water substitute during drying. Free radical releases are greatly delayed and even at high temperature the protein remains active. Trehalose, sucrose, maltose have shown to protect the protein structure during dehydration (Crowe et al. 1995).

Table- 4.1: Percentage of moisture at room temperature.

Name of the Sample	% of moisture
BSA	10.354
BSA formulated with mannitol	3.149
BSA formulated with ammonium sulfate	3.607
BSA formulated with trehalose	8.094
BSA formulated with maltodextrin	7.709

Analyzing the data obtained from TGA, it is found that BSA formulated with Mannitol contained less moisture content than samples formulated with other excipients. Trehalose formulation yielding hygroscopic and sticky products was found to have highest moisture content. After the analysis of the data through TGA, the samples are tested through the other techniques.

4.2 Aggregation study by Native Gel Electrophoresis

Aggregation is a common phenomenon which results from several types of reactions and uncontrolled folding. The chemical and physical transitions that a protein undergoes, changing from its native state to the unfolded state may result in aggregation, crystallization, degradation and denaturation. Aggregation can be regarded as an uncontrolled folding and unnatural folding, since both balance the exposed and buried hydrophobic surface areas of proteins. Hydrophobic interaction is the predominant driving force for both protein folding and aggregation. Aggregation occurs to minimize the thermodynamically unfavorable interactions between solvent and exposed hydrophobic residues of proteins (Yanli, 2005).

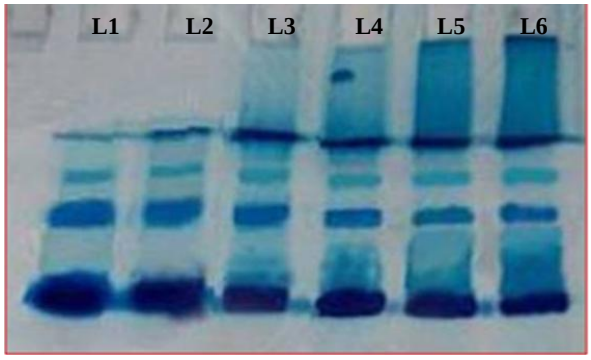

Fig. 4.2.1: It shows electrophoresis of BSA treated at different temperature for 60 minutes. Lane-1 shows native BSA i.e. control, Lane-2 shows BSA treated at 55°C, Lane-3 shows BSA treated at 60°C, Lane-4 shows BSA at 65°C, Lane-5 shows BSA at 70°C, Lane-6 shows BSA at 75°C

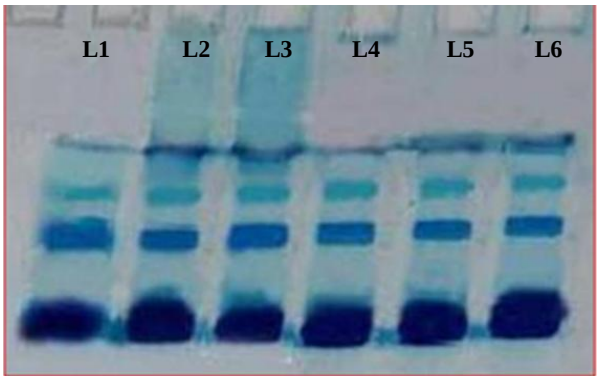

Fig. 4.2.2: It shows electrophoresis of BSA treated at 65°C for 10 minutes**.** Lane-1 shows native BSA i.e. control, Lane-2 shows BSA treated at 65°C for 10 min, Lane-3 shows BSA with maltodextrin, Lane-4 shows BSA with trehalose, Lane-5 shows BSA with ammonium sulfate, Lane-6 shows BSA with mannitol

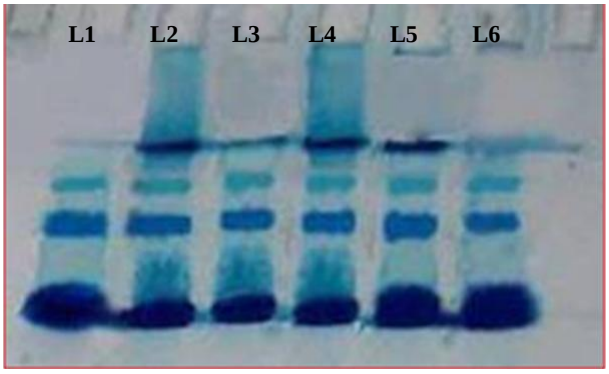

Fig. 4.2.3: Electrophoresis of BSA treated at 75°C for 10 minutes. Lane-1 shows native BSA i.e. control, Lane-2 shows BSA treated at 75°C for 10 min, Lane-3 shows BSA with trehalose, Lane-4 shows BSA with maltodextrin, Lane-5 shows BSA with ammonium sulfate, Lane-6 shows BSA with mannitol

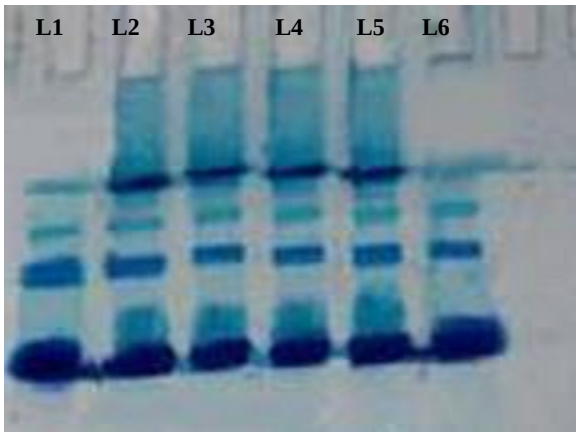

Fig. 4.2.4: Electrophoresis of BSA treated at 65°C for 60 minutes. Lane-1 shows native BSA i.e. control, Lane-2 shows BSA treated at 65°C for 60 min, Lane-3 shows BSA with trehalose, Lane-4 shows BSA with maltodextrin, Lane-5 shows BSA with ammonium sulfate, Lane-6 shows BSA with mannitol

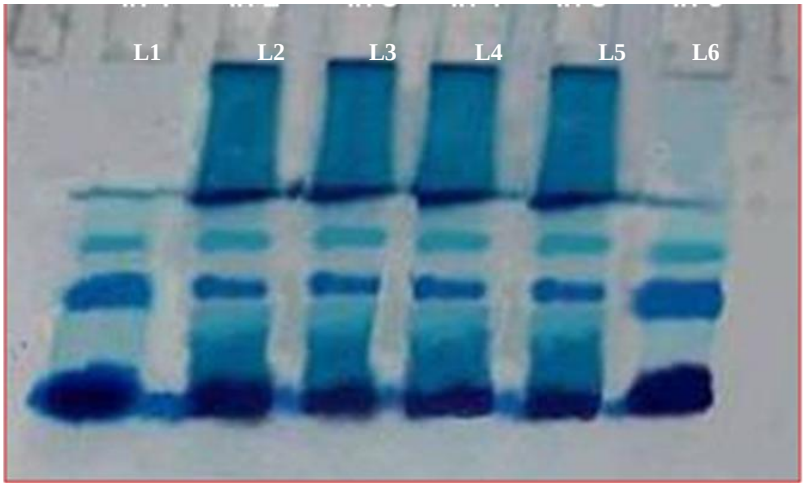

Fig. 4.2.5: Electrophoresis of BSA treated at temperature 75°C for 60 minutes**.** Lane-1 shows native BSA i.e. control, Lane-2 shows BSA treated at 75°C for 60 min, Lane-3 shows BSA with trehalose, Lane-4 shows BSA with maltodextrin, Lane-5 shows BSA with ammonium sulfate, Lane-6 shows BSA with mannitol

Comparing the above figures it is quite clear that Mannitol shows highest degree of protection as it produces the bands nearly equal to the bands of untreated control. Ammonium sulfate also shows protection against the thermal stress up to certain extent and trehalose does not show any significant protection where as maltodextrin forms similar type of band like the denatured ones hence shows no protection. Thus excipient decreases the aggregation of the protein and protects it against the thermal denaturation.

4.3 Fourier transfer spectroscopic study on protein

Characterization through FTIR

In FTIR spectroscopy, the light is directed onto the sample of interest, and the intensity is measured using an infrared detector. The intensity of light striking the detector is measured as a function of the mirror position, and this is then Fourier-transformed to produce a plot of intensity vs. wave number. We used FTIR analysis to evaluate changes in native secondary structure during thermal denaturation and spray drying of both control and formulated samples. Amide I and amide II bands are two major bands of the protein infrared spectrum out of the 9 characteristic bands given by peptide unit. The amide I band (between 1600 and 1700 cm^{-1}) is mainly associated with the C=O stretching vibration (70-85%) and is directly related to the backbone conformation. Amide II results from the N-H bending vibration (40-60%) and from the C-N stretching vibration (18-40%). Amide-I is the most intense absorption band in the proteins, that's why it is taken into consideration during analysis.

Structural analysis is done through FTIR. For analysis, the most sensitive Amide-I band is considered here. Samples were prepared according to method described in material and method section i.e. by heat treating them at constant temperature i.e. at 65°C and 75°C for different time intervals like: for 5, 10, 40 and 60 minutes in order to detect the change after thermal treatment. Bovine serum albumin basically under goes two types of structural changes after heat treatment (Kuznetsow et al., 1975). The first stage i.e. is heating up to 65°C is reversible while above to that temperature is regarded as the second stage which is irreversible. It is reported that β-sheets are formed when the protein is heated at 65°C and 70°C. Formation of β-sheets were concentration dependant. FTIR was carried out by considering the second derivative form of Amide-I region of the whole original spectrum. Denaturation of proteins is commonly associated with the alterations in the populations of the α-helix, β-sheet, and random coil structures. The α-helix is energetically less stable than the, β-sheet. As the percentages of denaturation increases the α-helix content decreases while in the reversible structural stage, some of the alpha-helices are transformed to random coils and aggregates are formed through the hydrogen bonding of beta-sheets between monomers. Increasing the temp. above the reversible stage, unfolding of the pocket exposing Cys-34 takes place, resulting in the formation of disulphide bridges which are covalent bonds, thus the stage is irreversible. The percentage of α-helices can be calculated by the relative area at 1655 cm^{-1}. The percentage of β-sheets can be calculated by the relative peak area at 1637

cm^{-1}. The percentage of β- turns can be calculated by adding the areas of all β-turn bands between 1670 and 1690 $cm^{-1.}$ The band area at 1648 cm^{-1} was assigned to random coil. By comparison, the result can be obtained. The secondary structure of BSA is composed of 67% helix, 10% turn, and 23% extended chain, and no β -sheet is contained (Murayama et al.,2004). Several workers have pointed out several theories regarding the secondary structure of the proteins i.e. particularly α-helix content in BSA. Studies indicate that the secondary structure of BSA contains about 68% - 50% alpha-helix and 16% -18% beta-sheet. But according to X-ray crystallography, there is no beta-sheet in the structure of native serum albumin. BSA is having 54% of α-helix measured by Optical rotatory dispersion. BSA is having 55% of α-helix measured by infrared spectroscopy. BSA is having 55-60% of α-helix measured by Raman spectroscopy. BSA is having 68% of α-helix measured circular dichriosm. Though different authors stated differently about the percentages of α-helix, but it is clear that BSA contains maximum helical region. In this study, the major concerned is given to α-helix.

In the double derivative form of the Amide-I band, specific wave numbers correspond to specific components of protein. These wave numbers give information about the particular band. According to Dong et.al. and Susi et al. 1624 $cm^{-1,}$ 1627 cm^{-1}, 1633 cm^{-1}, 1638 cm^{-1}, 1642 cm^{-1} are assigned to β –sheet, 1648 cm^{-1} is assigned to random coils, 1656 cm^{-1} is assigned to α-helix, 1663 cm^{-1} is assigned to 3_{10} helix, 1667 cm^{-1} is assigned to β-turns. For the data analysis of FTIR, several methods are used, like: relative area calculation (for quantifying the percentage of structural components), Curve fitting method and Gauss peaks can be drawn. In this study, due to unavailability of software, all the methods could not be practiced. We have analyzed the second derivative spectra of the deconvoluted form of the amide-I region of the protein. During result analysis we got significant difference in the no. of bands, band shapes, appearance and disappearance of bands of control, the thermally denatured native and formulated proteins(after spray drying). For the native state, the band is fairly symmetric and has a peak maximum around 1655 cm^{-1} which corresponds to alpha-helical structure and 1660-1666 cm^{-1} corresponding to 3_{10} helix. 3_{10} helix is rarely found in the proteins. It is like helical portion of the protein in which the 1^{st} atom is paired with 3^{rd} atom not like 1^{st} with the 4^{th}. In contrast, the denatured proteins show no peaks at the region of the 1655 cm^{-1} and additional maximum peak between 1690 and 1667 cm^{-1}, indicative of the predominance of beta-sheet and beta-turn structures.

Using Ammonium sulfate as an excipient

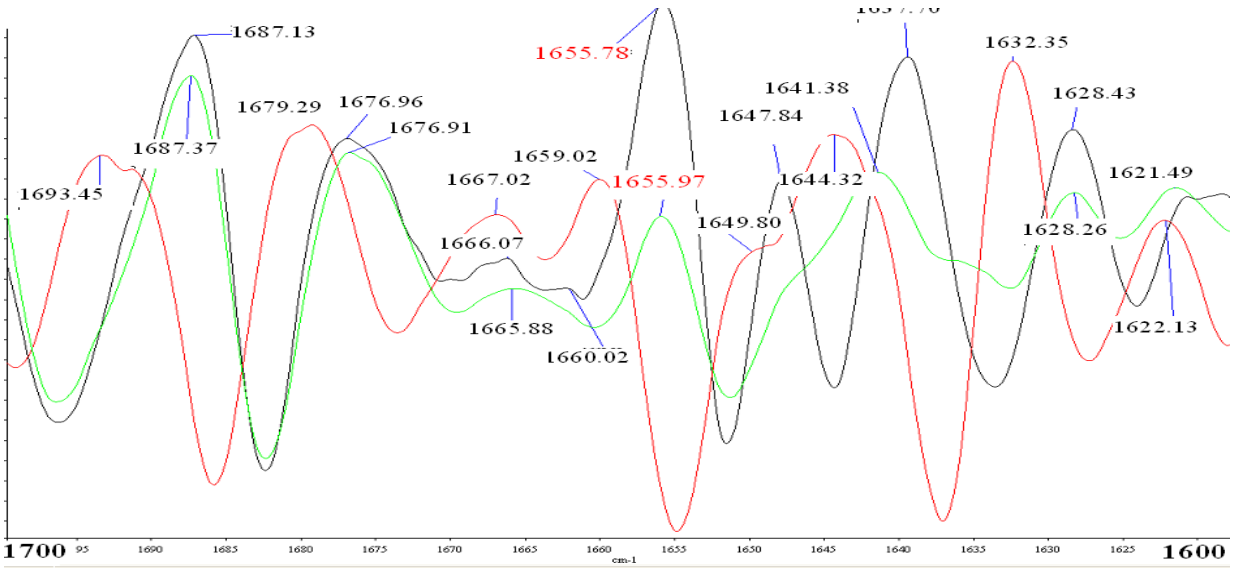

Fig. 4.3.1 FTIR plots of native protein (black line i.e. reference at room temp.) protein with out excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 65°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

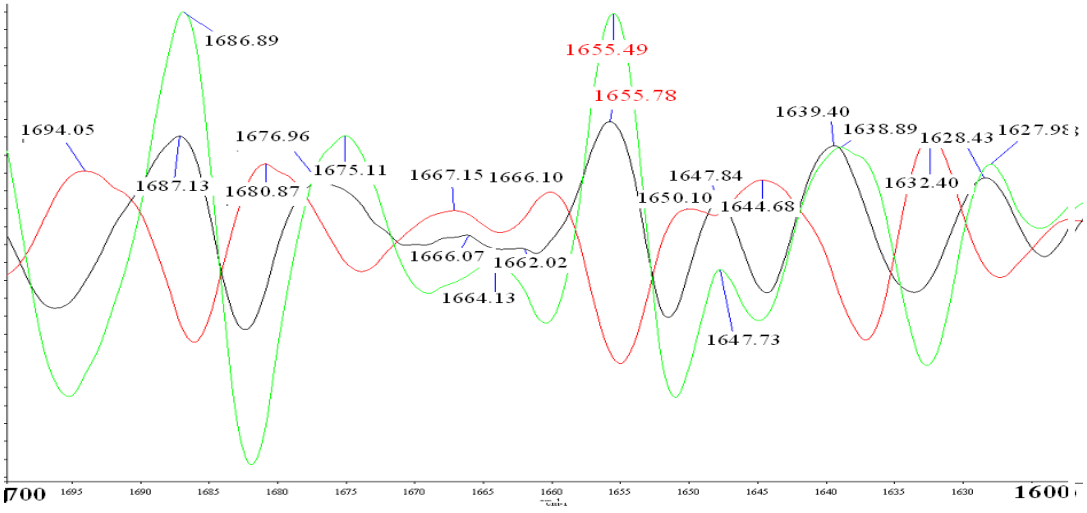

Fig. 4.3.2 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 65°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

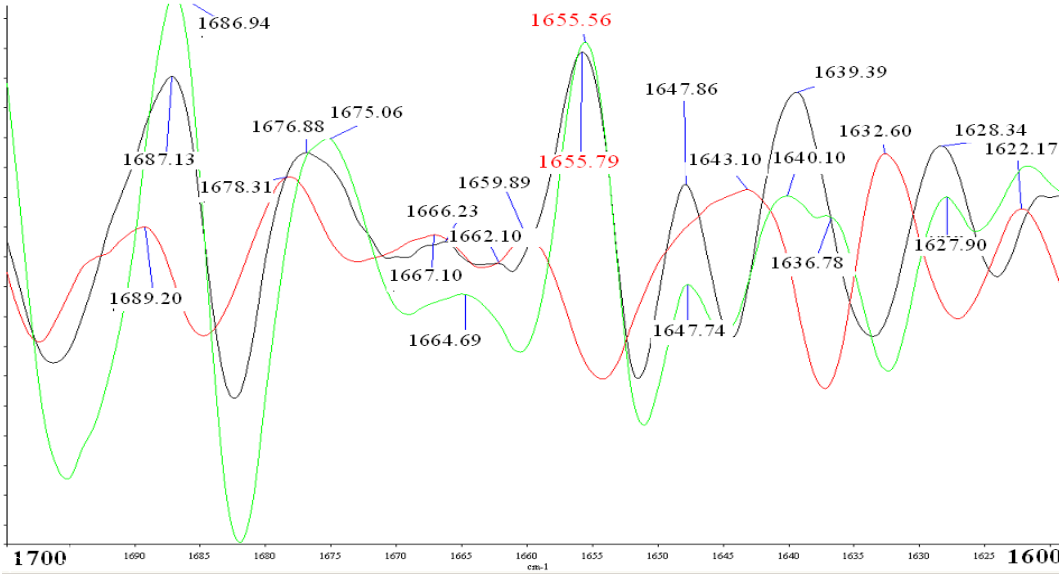

Fig. 4.3.3 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 65°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

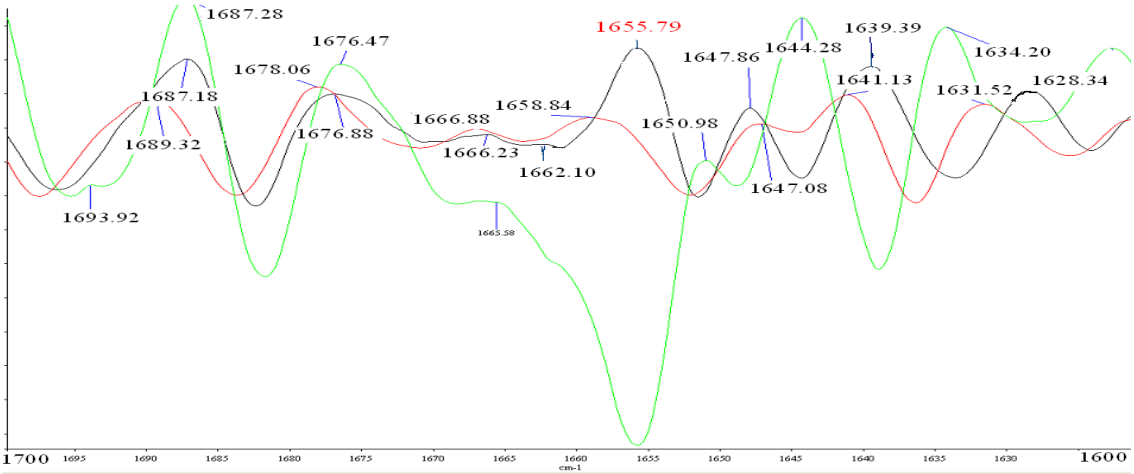

Fig. 4.3.4 FTIR plots of native protein (black line i.e. reference at room temp.) protein with out excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 65°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

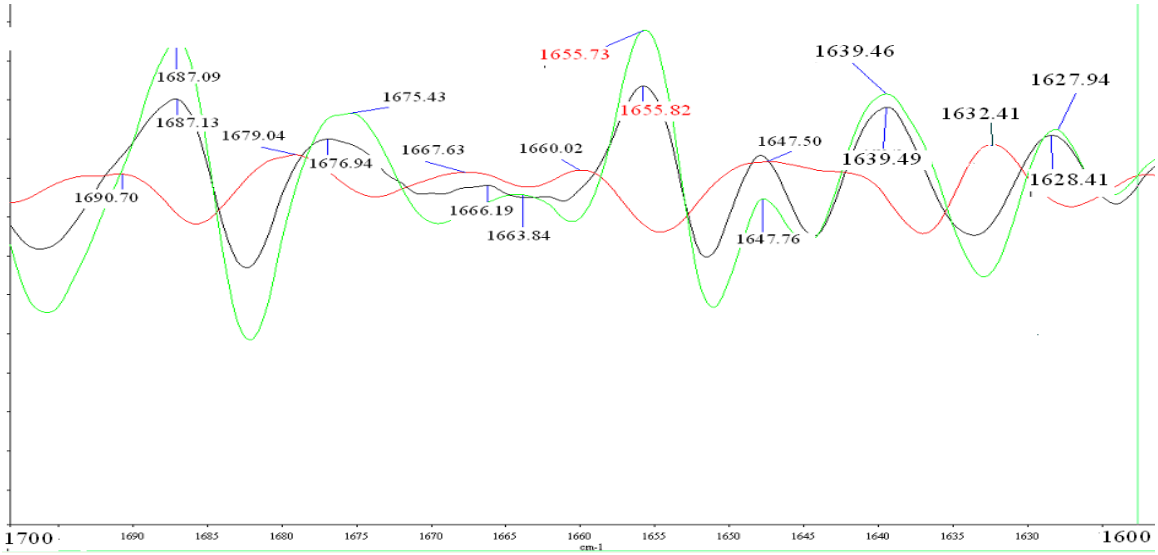

Fig. 4.3.5 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 75°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

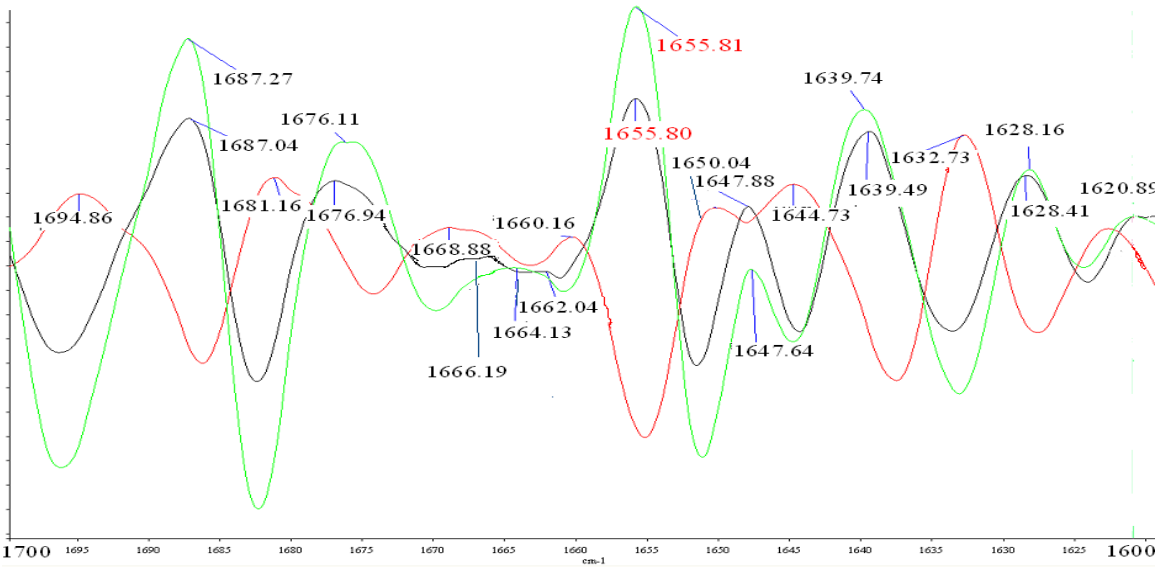

Fig. 4.3.6 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 75°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600cm^{-1} and Y-axis: absorbance)

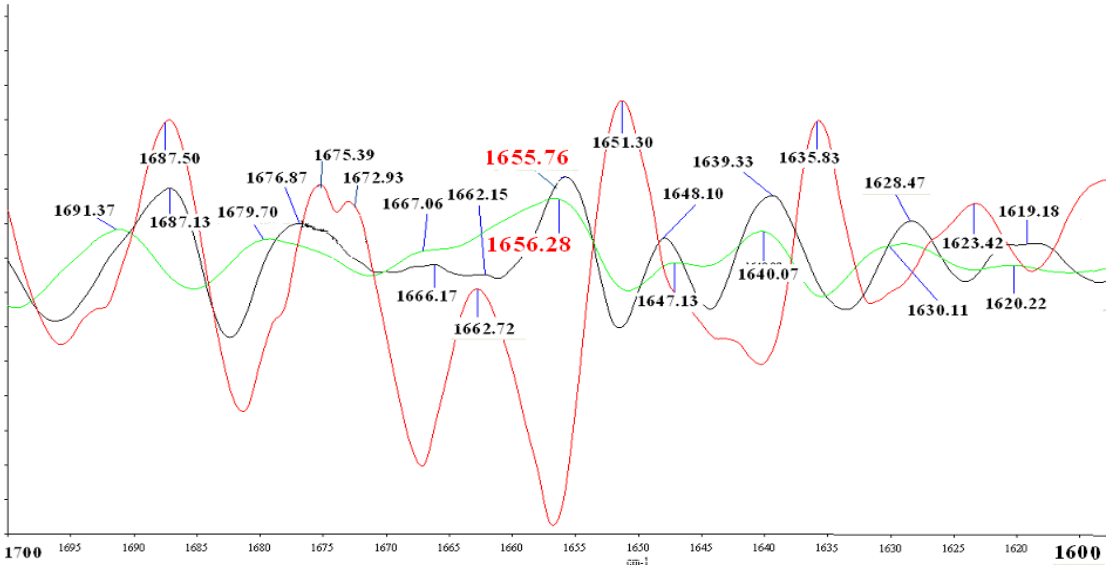

Fig. 4.3.7 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 75°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

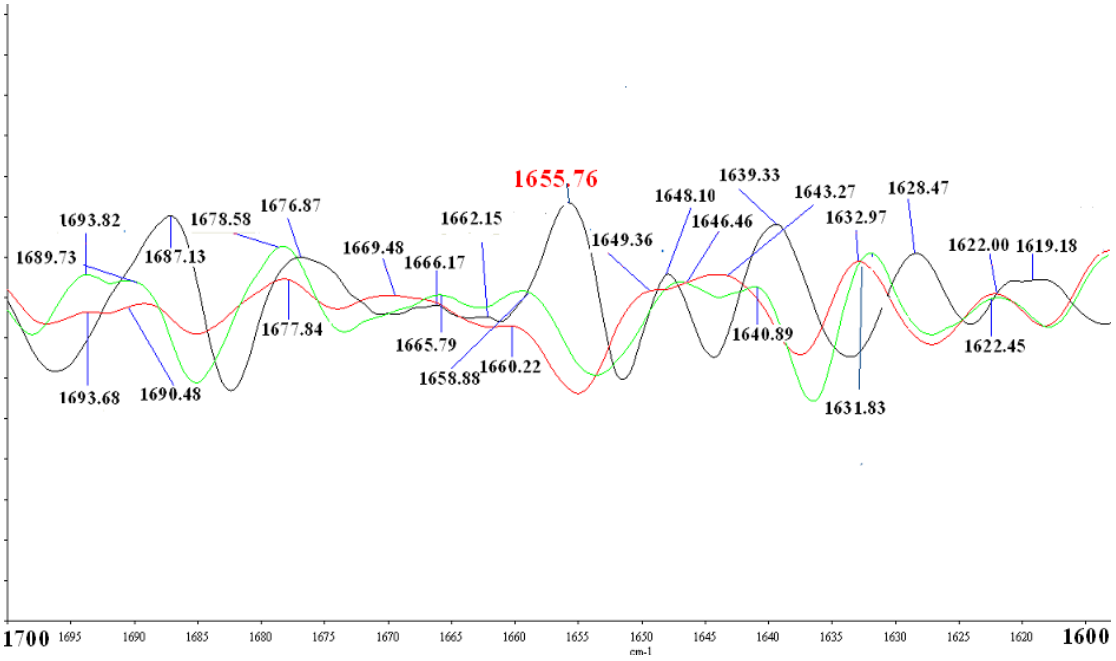

Fig. 4.3.8 FTIR plots of native protein (black line i.e. reference at room temp.) protein with out excipient heat treated (red line) and formulated sample (green line- spray dried with ammonium sulfate at 75°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

Total 8 peaks are there at different regions of the native protein graph i.e. at room temperature. By comparing each peak, (Fig. 4.3.1) one can clearly conclude that formulated sample producing the same types of peaks as that of the control. There is neither change in the peaks position nor in the peak numbers.

Fig. 4.3.2 heat treated protein lacks peaks at the region of 1655cm^{-1} which is related to the α-helix and also peaks are shifted from its original position can be marked. The peak found in the region of 1687 cm^{-1} is shifted to 1693 cm^{-1},1676 cm^{-1} to 1679 cm^{-1} and 1641 cm^{-1} to 1644 $cm^{-1.}$ But in case of formulated ones, it does protect the band at 1655 cm^{-1} and there is no shift of peaks and the peak numbers and position are identical to that of control. Fig. 4.3.3 Treated protein lacks α-helical peak at the region of 1655 cm^{-1} and also peaks are shifted from its original position can be marked. But in case of formulated ones, it gives protection to the band at 1655 cm^{-1} and there is no shift of peaks and the peak numbers and pertain are identical to that of control. In Fig. 4.3.4 preserves all the peaks and there are no changes in the original position. In case of the heat treated samples, α-helical peak is absent indicating that after heat treatment the α-helix is strongly affected (Liao et al., 2002) and the formulated sample is capable to protect the protein helicity. Fig. 4.3.4 Formulated sample can not able to protect the protein structural component at 1655 cm^{-1} and also clear shift of the bands and new formation of band at 1693 cm^{-1}. It produces same type of result to that of heat treated one. Hence it can be seen that at 65 ^{o}C for 60 minute i.e. protein when exposed to temperature for more time, excipient cannot be able to give protection.

Fig. 4.3.5 Heat treated protein lacks peaks at the region of 1655cm^{-1} which is related to the α-helix and also peaks are shifted from its original position can be marked. But in case of formulated ones, it shields the band at 1655 cm^{-1} and there is no shift of peaks and the peak numbers and pertain are identical to that of control one. Fig. 4.3.6 Protein lacks peaks at the region of 1655cm^{-1} which is related to the α-helix and also peaks are shifted from its original position after heat treatment. But in case of formulated ones, it preserves the band at 1655 cm^{-1} and all the peaks are remained conserved. Fig. 4.3 7 Protein lacks peaks at the region of 1655cm^{-1} which is related to the α-helix and also peaks are shifted from its original position after heat treatment. But in case of formulated ones, it preserves the band at 1655 cm^{-1} and all the peaks are remained intact. Fig.4.3.8 Protein lacks peaks at the region of 1655cm^{-1} which is related to the α-helix and also peaks are shifted from its original position after heat treatment and in case of formulated ones, it also does not able to protect the protein when

protein exposed for longer time i.e. for 60 min as the band at 1655 cm^{-1} is also absent and and the peak shifts and another peak at β-sheet region is created.

From the above discussion this can be clearly concluded that this excipient protects the α-helix at 1655 cm^{-1} and 3_{10} helix at 1665 cm^{-1} and β-turn at 1687 cm^{-1} like that of native protein where as the denatured one shows additional peak as β-sheet at 1693 cm^{-1} and β-turn at 1667 cm^{-1} respectively. This indicates that ammonium sulfate formulated samples are giving the protection at 65 ^{o}C and also 75 ^{o}C but up to 40 minutes. But when the protein is getting exposed to those temperatures for more time i.e. for 60 minute it can not give protection by producing nearly equal peak, i.e. lacking the helical band at the region of 1655 cm^{-1} and creation of new bands at the region of 1693 cm^{-1} which is related to like that of heat treated sample β-sheet region (Dong et al.,1994)

Using Mannitol as an excipient

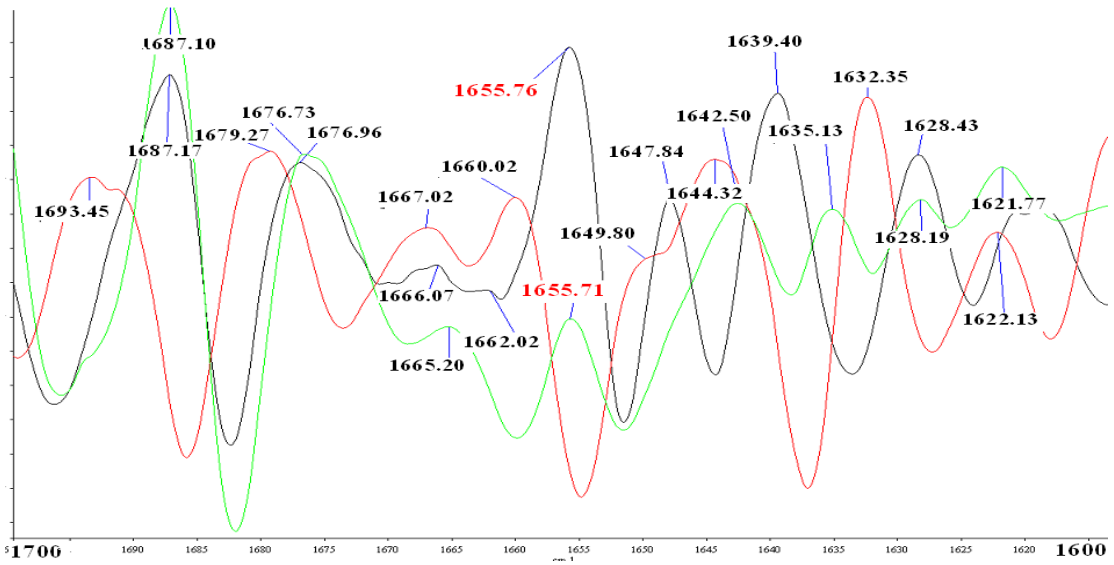

Fig. 4.3.9 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 65 $^{\circ}$C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.9 the formulated sample conserves the peaks corresponding to β-turn at 1687 cm^{-1} 3_{10} helix at 1665 cm^{-1} α-helix at 1655 cm^{-1} and β-sheet at 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1693 cm^{-1} and β-turn at 1667 cm^{-1} and β-sheet at 1632 cm^{-1}.

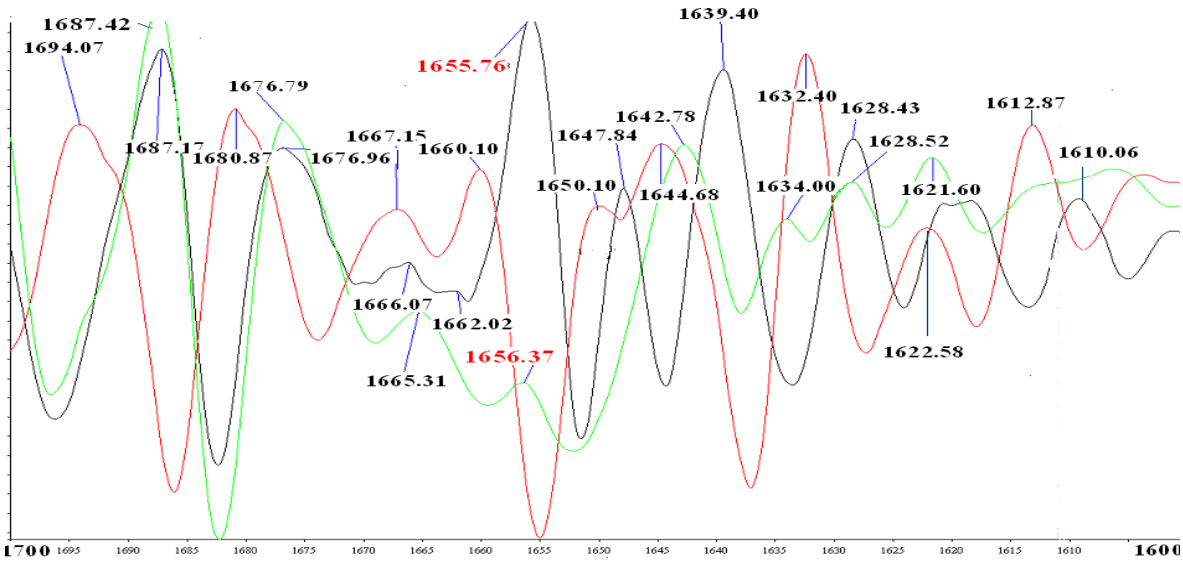

Fig. 4.3.10 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 65°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.10, the formulated sample conserves the peaks corresponding to β-turn at 1687 $cm^{-1}$and 1676 cm^{-1} 3_{10} helix at 1665 cm^{-1} α-helix at 1655 cm^{-1} and β-sheet at 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1693 cm^{-1}, 1642 cm^{-1}, 1632 cm^{-1}, and β-turn at 1680 cm^{-1}, 1667 cm^{-1}.

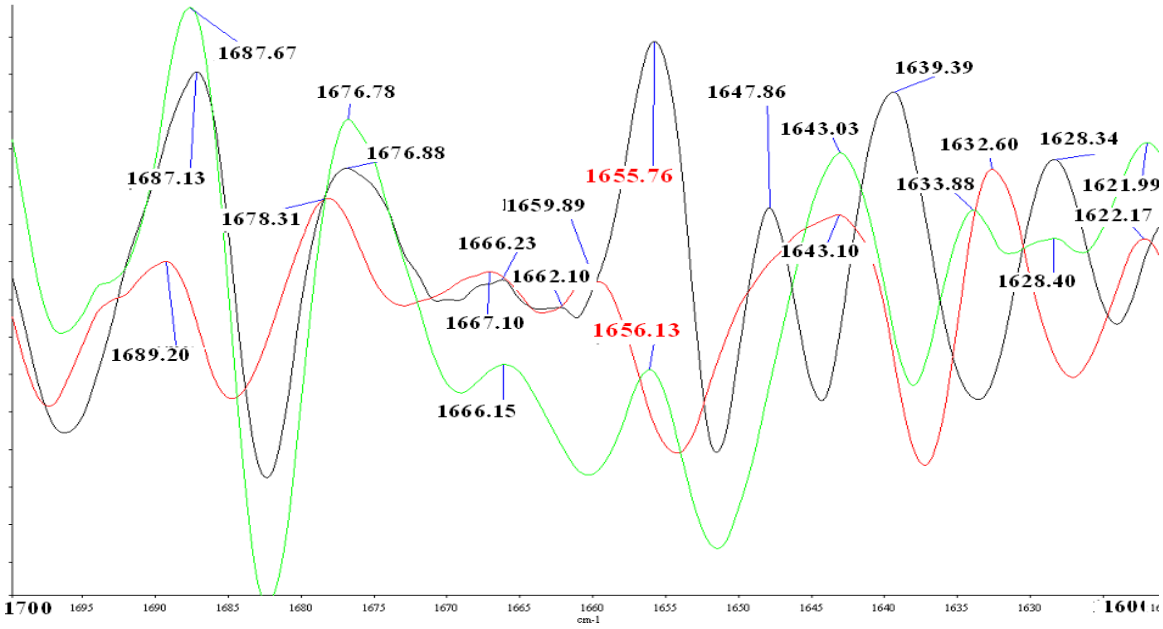

Fig: 4.3. 11 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 65°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig-4.3.11, the formulated sample conserves the peaks corresponding to β-turn at 1687 cm^{-1} , 1676 cm^{-1}, 3_{10} helix at 1666 cm^{-1} α-helix at 1655 cm^{-1} and β-sheet at 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1689 cm^{-1} and β-turn at 1667 cm^{-1} and β-sheet at 1632 cm^{-1}.

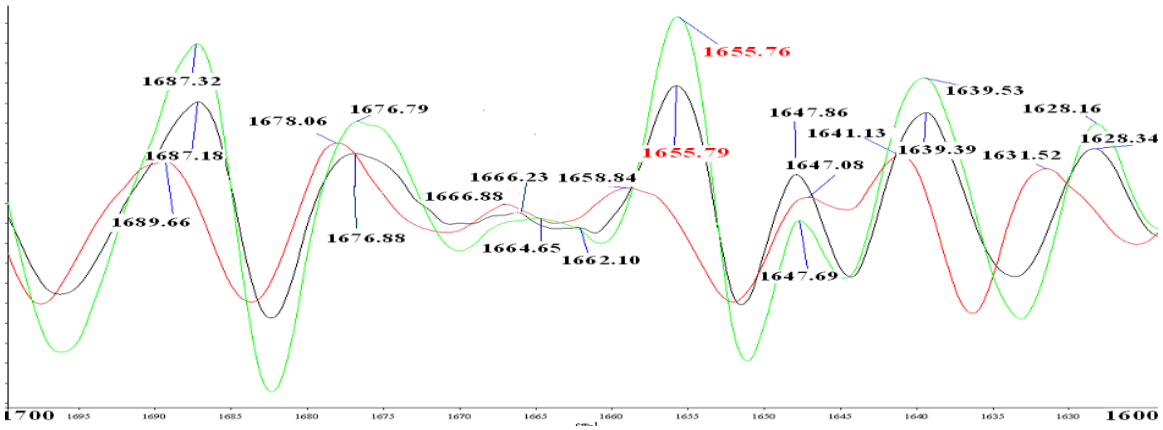

Fig. 4.3.12 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 65°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig: 4.3.12, the formulated sample conserves the peaks corresponding to β-turn at 1687 cm^{-1}, α-helix at 1655 cm^{-1} and β-sheet at 1639 cm^{-1}, 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1689 cm^{-1} and β-turn at 1667 cm^{-1} and β-sheet at 1641 cm^{-1}, 1632 cm^{-1}.

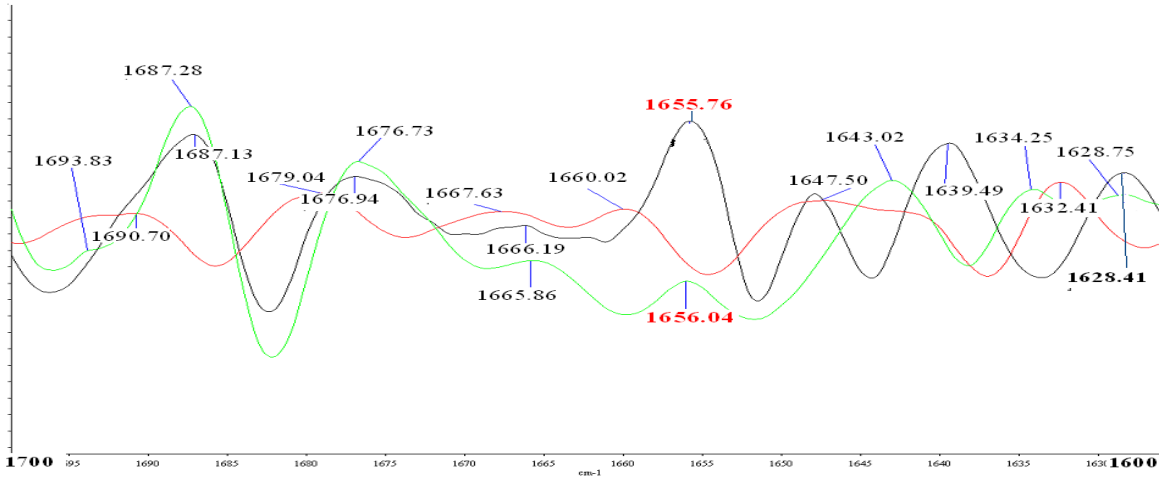

Fig. 4.3.13 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 75°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.13, the formulated sample conserves the peaks corresponding to β-turn at 1687 cm^{-1} , 1676 cm^{-1}, and β-sheet at 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-turn at 1679 cm^{-1} and 3_{10} helix at 1660 cm^{-1} and β-sheet at 1632 cm^{-1}.

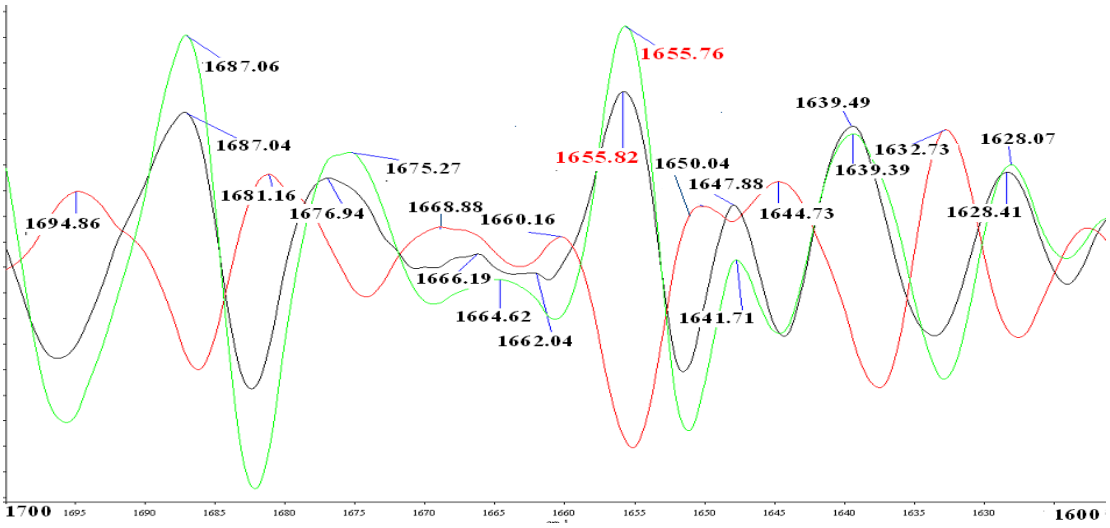

Fig. 4.3.14 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 75°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.14, the formulated sample conserves the peaks corresponding to β-turn at 1687 cm^{-1} and 1676 cm^{-1} .α-helix at 1655 cm^{-1} and β-sheet at 1639 and 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1694, 1644 and 1632 cm^{-1}. β-turn at 1681 and 1667 cm^{-1} and random coil at 1650 cm^{-1} .

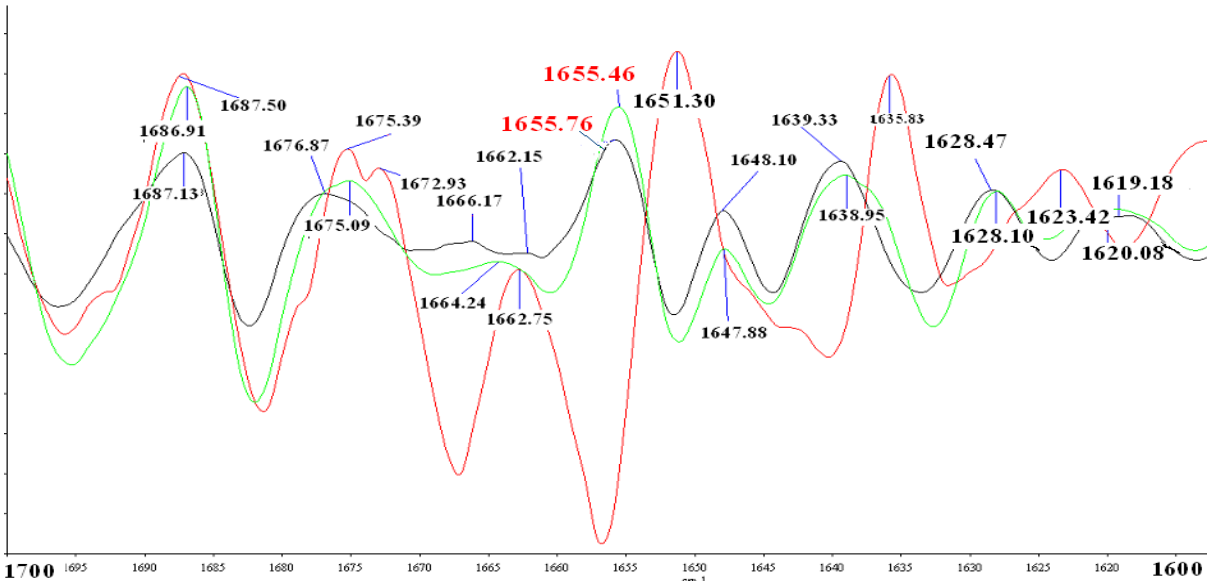

Fig. 4.3.15 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 75°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.15, the formulated sample conserves the peaks corresponding to α-helix at 1655 cm^{-1} random coil at 1648 cm^{-1} and β-sheet at 1639 and 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-turn at 1675 cm^{-1} and β-sheet at 1635 and 1632 cm^{-1}.

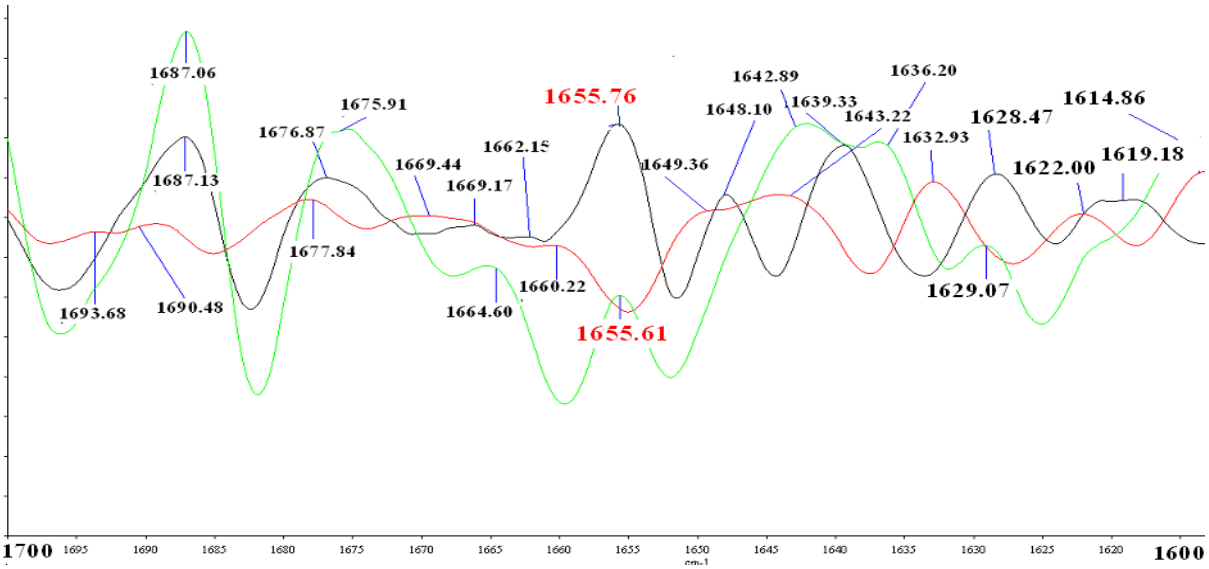

Fig. 4.3.16 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with mannitol at 75°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.16, the formulated sample conserves the peaks corresponding to β-turn at 1687 & 1676 cm^{-1} , α-helix at 1655 cm^{-1} and β-sheet at,1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1643 & 1632 cm^{-1} and random coil at 1649cm^{-1}.

The double derivative of the deconvoluted form of Amide-I was taken for each sample. By analyzing each peak of the graph it can be marked the effect of excipient on the heat treated samples. After analyzing the FTIR plots for the protein formulation using mannitol as an excipient, it concluded that this excipient (mannitol) conserves the peaks that correspond to the β-turn at 1687 cm^{-1} , 1676 cm^{-1}, 3_{10} helix at 1665 cm^{-1}, α-helix at 1655 cm^{-1} and β-sheet 1639 cm^{-1} 1628 cm^{-1} and random coil at 1650 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1693 cm^{-1} ,1643 cm^{-1} , 1632 $cm^{-1}$and β-turn at 1681 cm^{-1}, 1675 cm^{-1} , 1667 cm^{-1} and random coil at 1650 cm^{-1} . Both at 75°C and 65°C heat treatment it can protect upto an exposure time of 1hr. thus conferring protection to the protein molecule and hence a good excipient.

Using Maltodextrin as an excipient

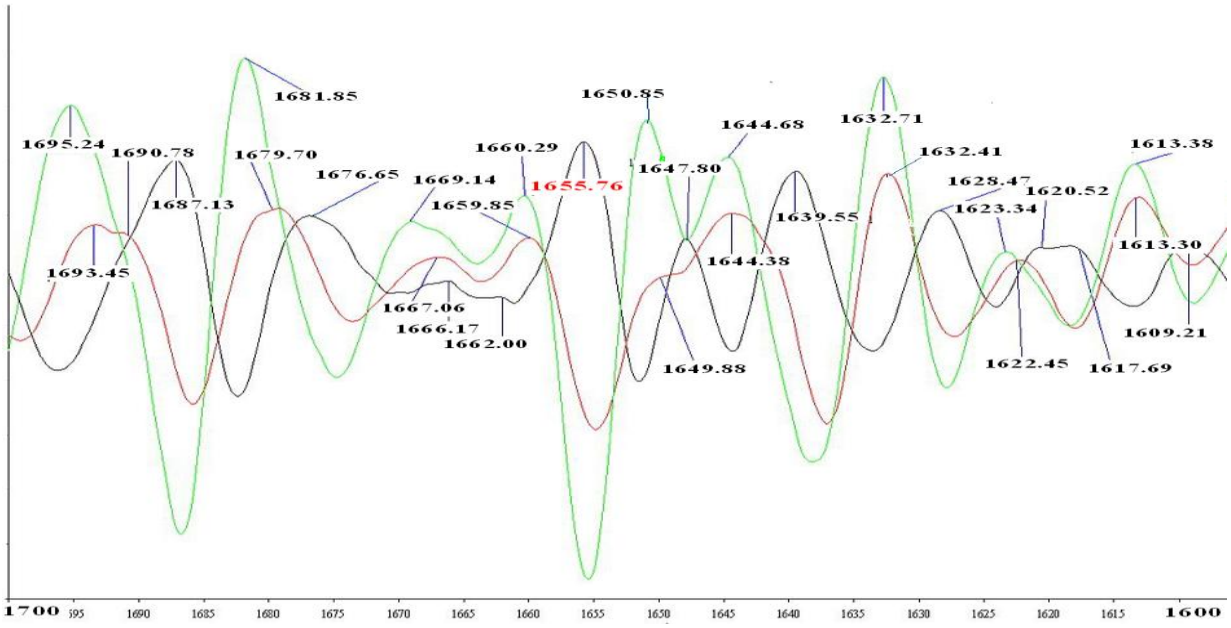

Fig. 4.3.17 FTIR plots of native protein (black line i.e. reference at room temp.) protein with out excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 65°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance).

Observing the FTIR plots for formulated protein using maltodextrin as an excipient (Fig-4.3.17) it is clearly visible that, the plots of thermal denatured native protein goes side by side with that of the formulated protein. In the formulated protein the basic peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure.

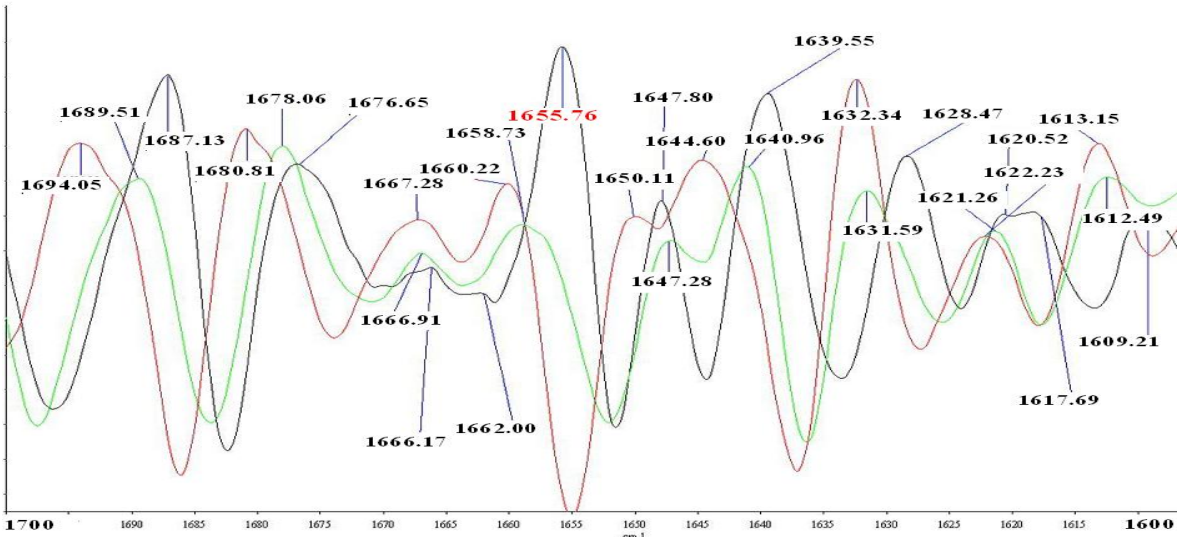

Fig.4.3.18 FTIR plots of native protein (black line i.e. reference at room temp.) protein with out excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 65°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance).

In Fig. 4.3.18 all the three plots i.e. of control, native and formulated samples have similar kind of peaks which is clearly visible. In the formulated protein the basic peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure.

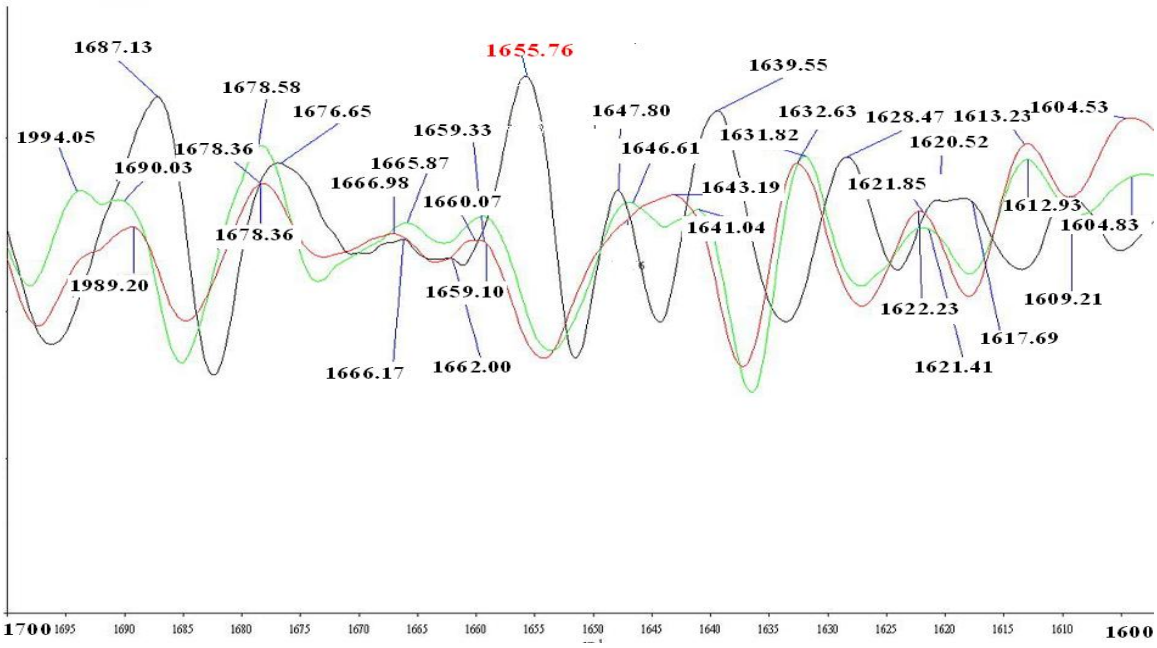

Fig. 4.3.19 FTIR plots of native protein (black line i.e. reference at room temp.) protein with out excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 65°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance).

Analyzing the result for Fig. 4.3.19, we observed the similar type of situation as that of the above two cases. The plots of thermal denatured native protein goes side by side with that of the formulated protein, whereas control shows different peaks than that of the other two. In the formulated protein the basic peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure.

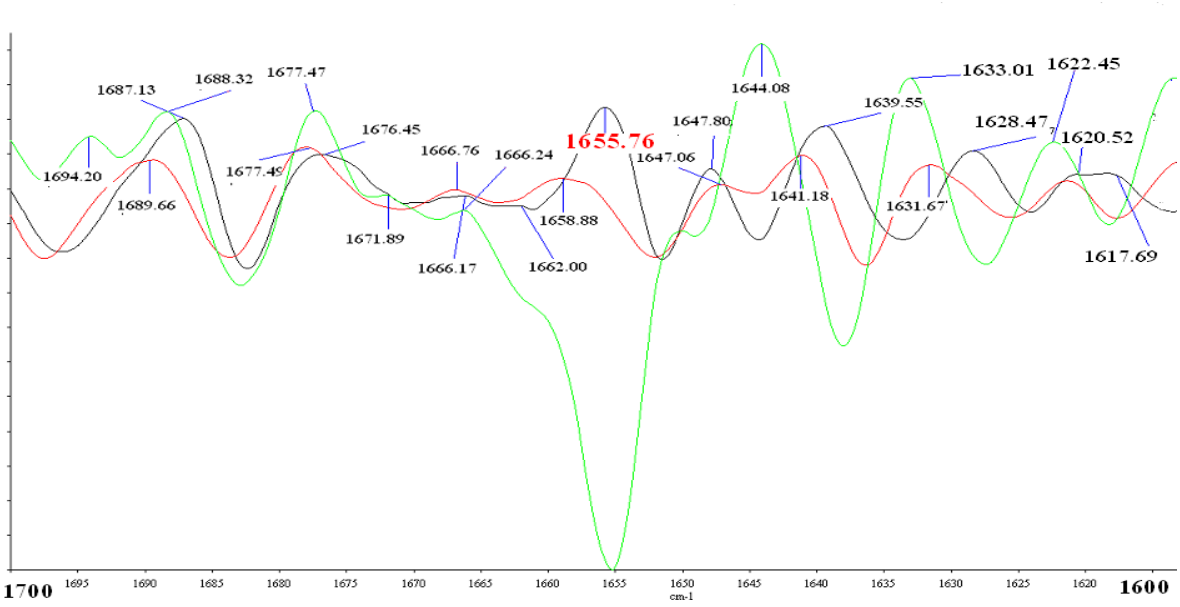

Fig. 4.3.20 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 65°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance).

For formulated sample in place of a peak corresponding to α-helix at 1655 cm^{-1} we got a trough over here, which is a clear cut indication that α-helix is not protected. Also the plots of thermal denatured native protein goes side by side with that of the formulated protein. So the excipient is not working in the specified temperature for the specific time of exposure.

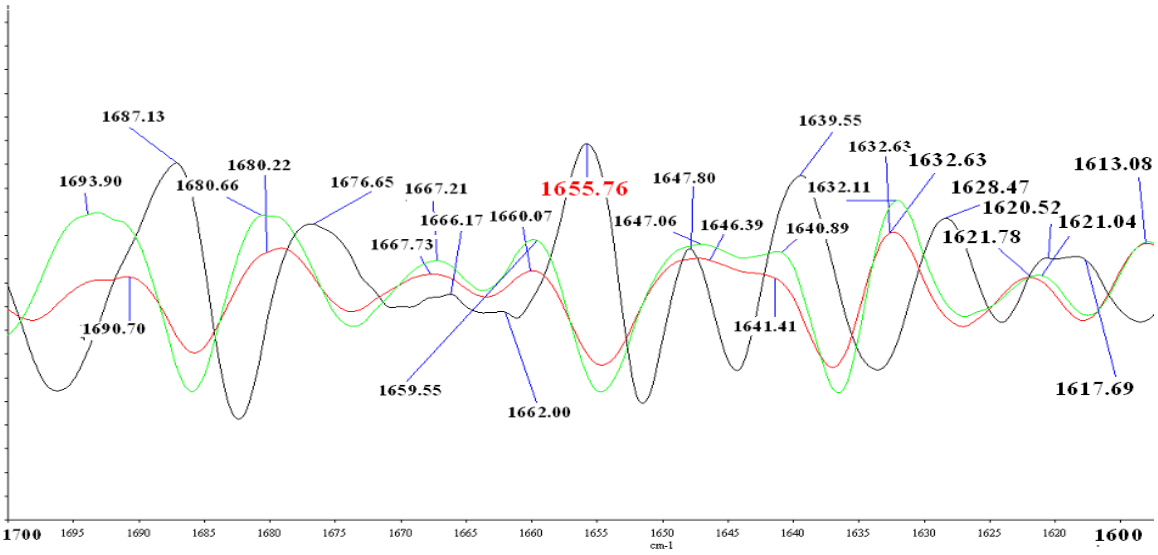

Fig. 4.3.21 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 75°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance).

Formulated and heat treated protein lacks peaks at the region of 1655 cm^{-1} (Fig.4.3.21) which is related to the α-helix and also peaks are shifted from its original position that can be marked. The peak found in the region of 1687 cm^{-1} is shifted to 1693 cm^{-1}, 1676 cm^{-1} to 1679 cm^{-1} and 1641 cm^{-1} to 1644 cm^{-1}. In case of formulated ones, it does not protect the band at 1655 cm^{-1} and there is shift of peaks and the peak numbers and hence the excipient fails to protect the protein in specified conditions.

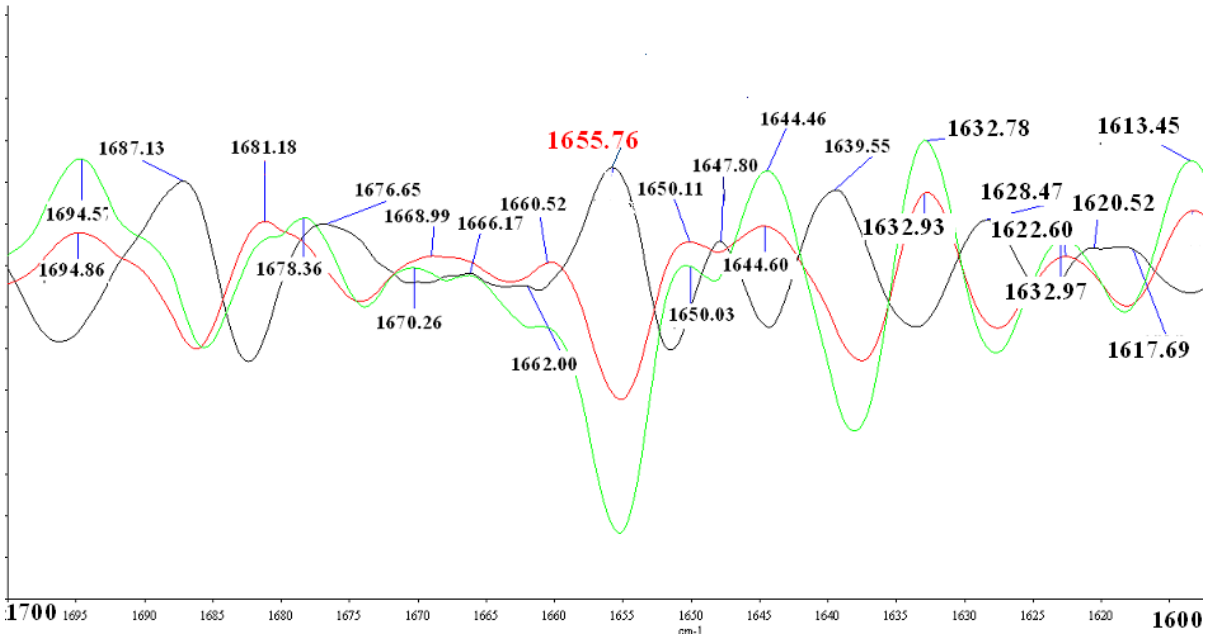

Fig 4.3.22 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 75°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

As per Fig. 4.3.22, we observed the similar type of situation as that of the above two cases. The plots of thermal denatured native protein goes side by side with that of the formulated protein, whereas control shows different peaks than that of the other two. In the formulated protein the basic peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure

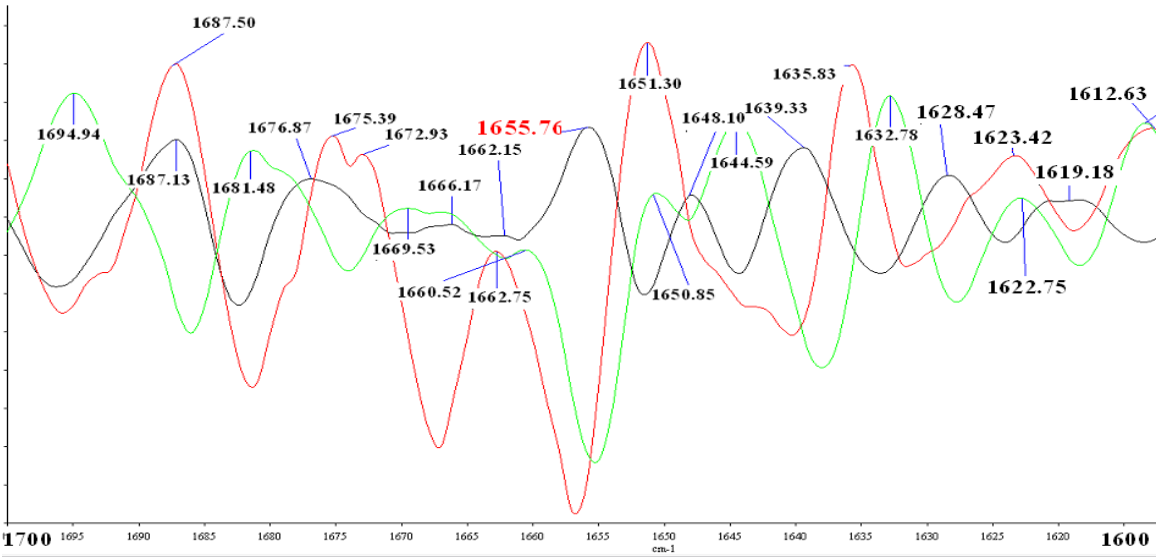

Fig. 4.3.23 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 75°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

In the above figure we got totally irregular results. For the higher wave numbers native denatured sample showed similar peaks with that of control one, where as for lower wave number native denatured sample shows similar peaks as that of formulated one, but the very basic for stability of formulated protein is the peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure

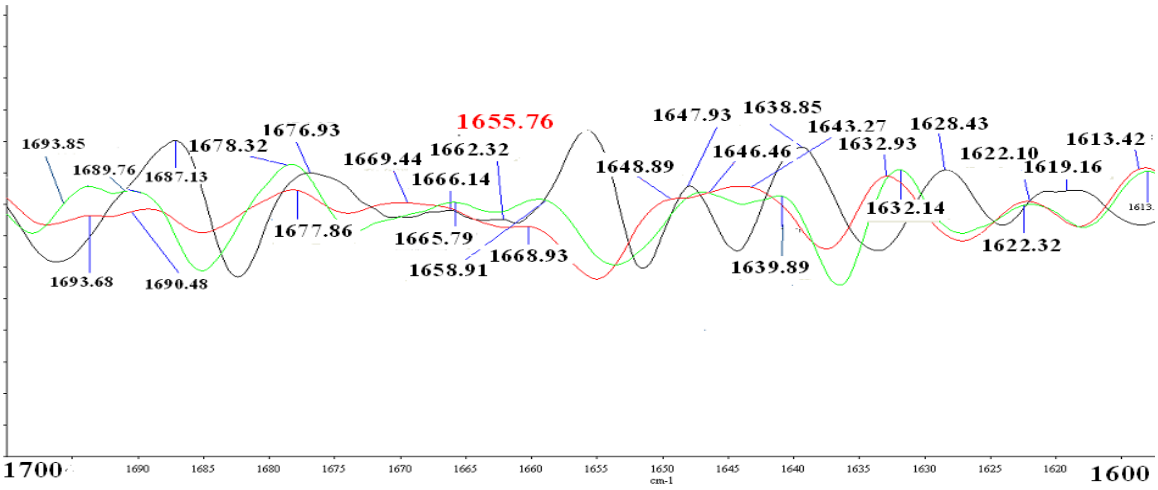

Fig. 4.3.24 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with maltodextrin at 75°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance

For this particular plot we observed at higher wave number all the three plots showing similar type of peaks but for lower wave numbers native denatured sample shows similar peaks as that of formulated one. And again the vital peak is missing i.e. α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure.

Now coming to the overall result analysis of the formulated sample using maltodextrin as excipient for the specific temperature and specified time of exposure to heat we can concluded that, maltodextrin can not protect the basic α-helix at 1655 cm^{-1}. Neither it shows protection for higher time of exposure nor for lower time of exposure. It neither protects at 75°C nor at 65°C. So it is not a better option to be used as an excipient.

Using Trehalose as an excipient:

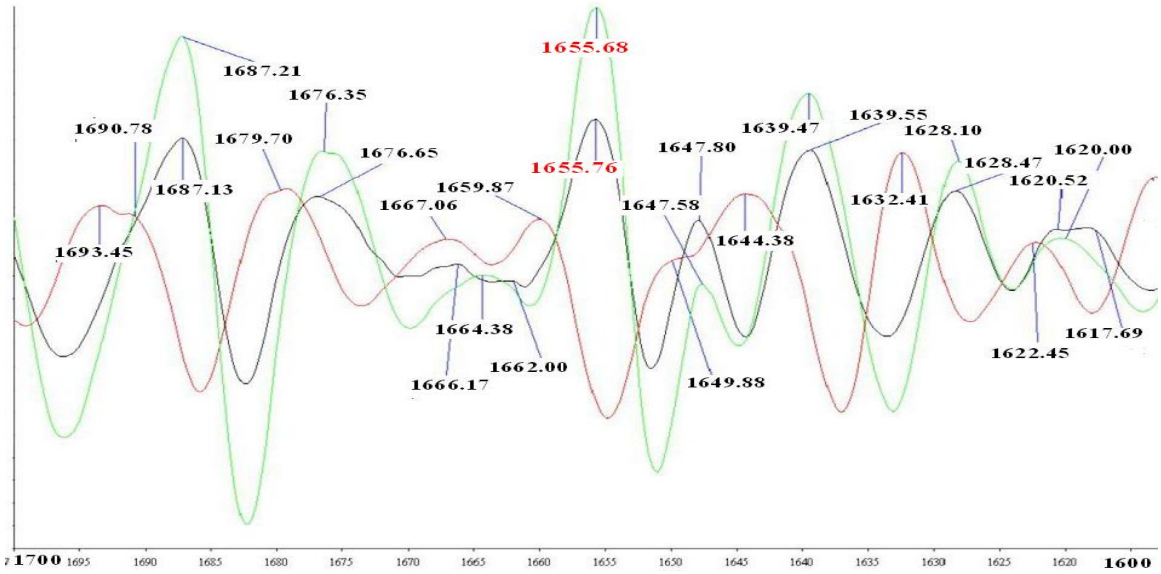

Fig. 4.3.25 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 65°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance).

As per Fig4.3.25, the formulated sample conserves the peaks corresponding β-turn at 1687 cm^{-1} , 1679 cm^{-1} and 1676 cm^{-1} . α-helix at 1655 cm^{-1} and random coil at 1649 cm^{-1} . β-sheet at 1639 cm^{-1} & 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1693 cm^{-1}, 1644 cm^{-1} and1632 cm^{-1} and β-turn at 1679 cm^{-1} and 1667 cm^{-1}. Thus excipient confers protection.

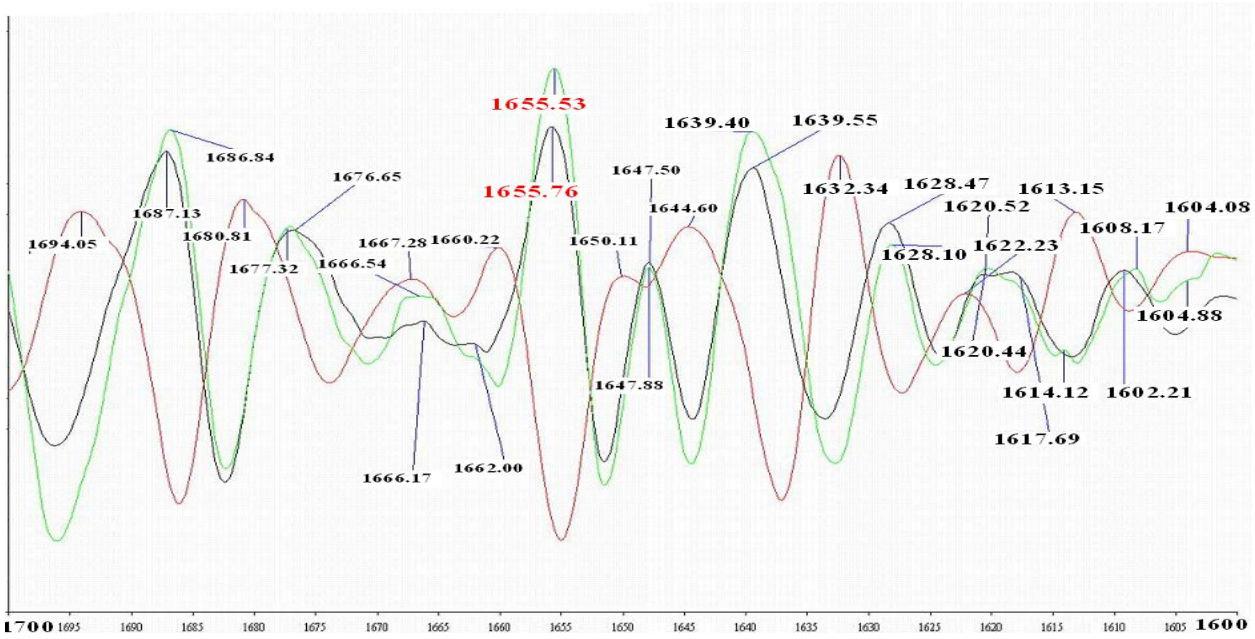

Fig. 4.3.26 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 65°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

Coming to Fig. 4.3.26, the formulated sample conserves the peaks corresponding β-turn at 1687 cm^{-1} and 1676 cm^{-1}, α-helix at 1655 cm^{-1}, 3_{10} helix at 1666 cm^{-1} random coil at 1647 cm^{-1}. β-sheet at 1639 cm^{-1} & 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1694 cm^{-1}, 1644 cm^{-1} and1632 cm^{-1} and β-turn at 1680 $cm^{-1}$and 1667 cm^{-1}, random coil at 1650 cm^{-1}. Thus excipient confers protection.

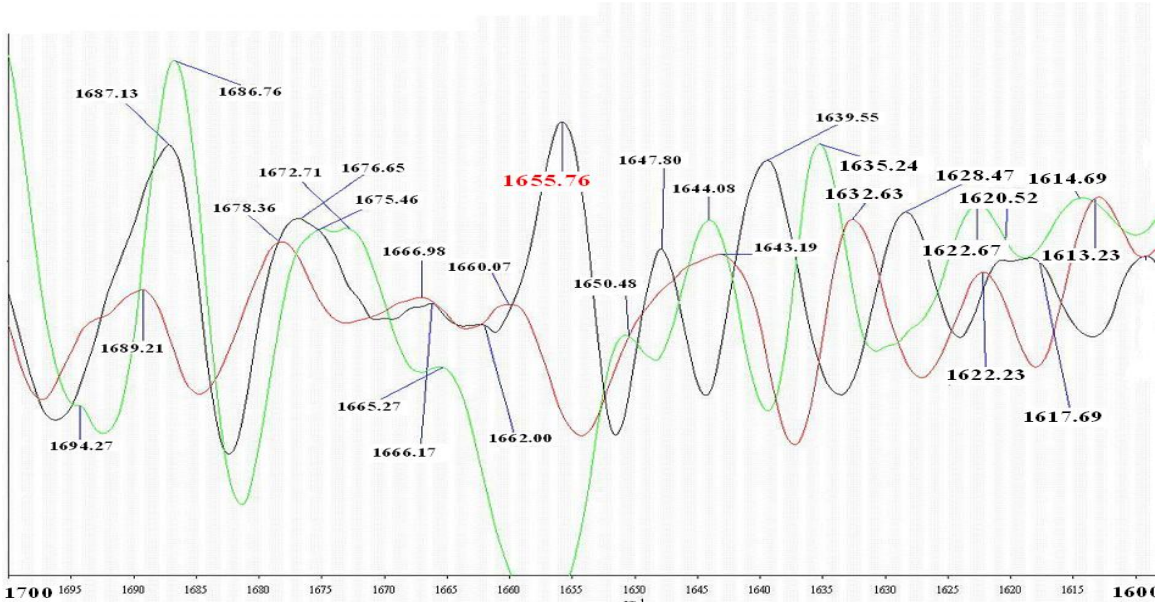

Fig. 4.3.27 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 65°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

In the above figure we got peculiar results. For the higher wave numbers formulated sample showed similar peaks with that of control one, where as for lower wave number plots for each of the sample showed unique peaks and the very basic for stability of formulated protein is the peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure

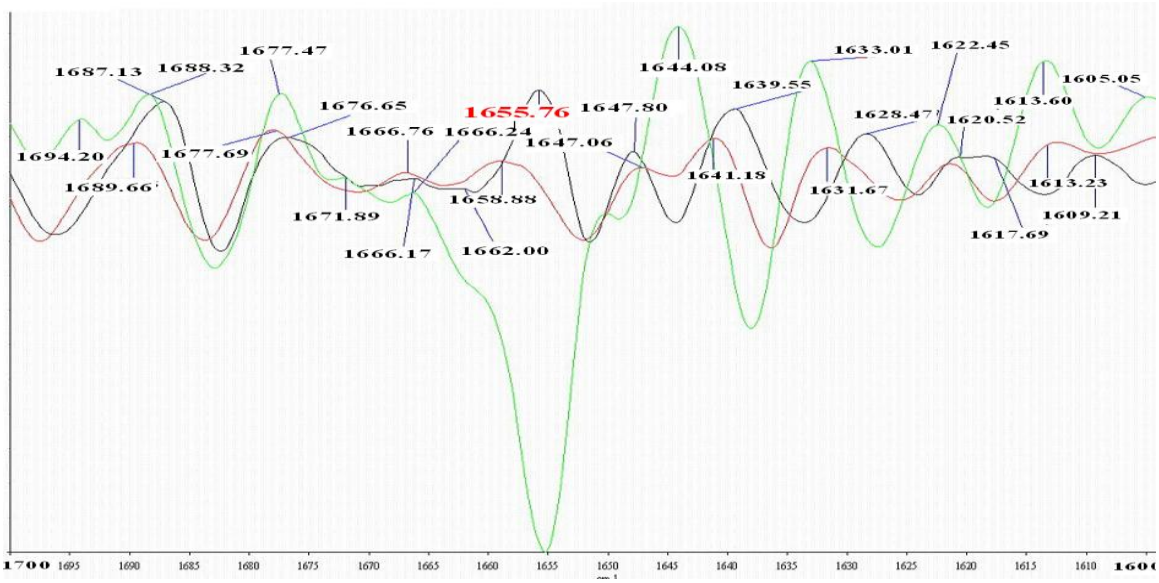

Fig. 4.3.28 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 65°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600cm^{-1} and Y-axis: absorbance)

For formulated sample in place of a peak corresponding to α-helix at 1655 cm^{-1} we got a trough over here, which is a clear cut indication that α-helix is not protected. Also all the three plots of the corresponding samples thermal goes side by side. So the excipient is not working in the specified temperature for the specific time of exposure

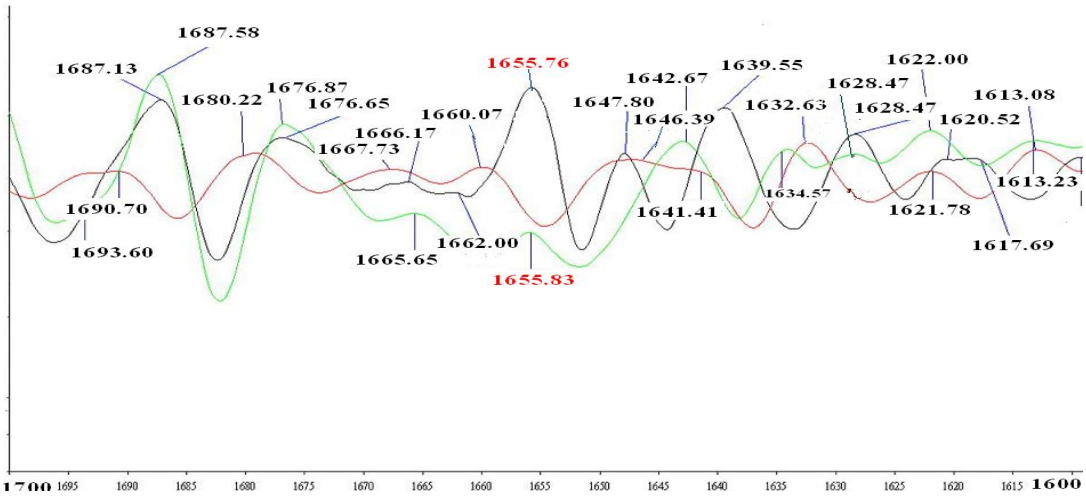

Fig. 4.3.29 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 75°C for 5 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

Discussing the result for the plot given in Fig. 4.3.25, the formulated sample conserves the peaks corresponding β-turn at 1687 cm^{-1} and 1676 cm^{-1}, α-helix at 1655 cm^{-1}, β-sheet at 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1640 cm^{-1} and 1632 cm^{-1} and β-turn at 1680 cm^{-1} and 1667 cm^{-1} . Thus excipient confers protection

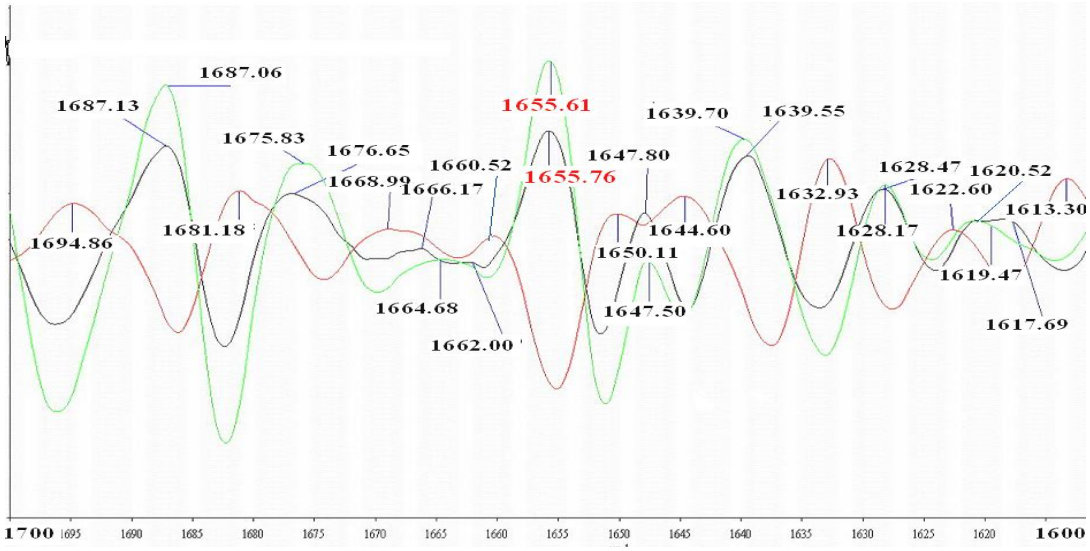

Fig:4.3.30 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 75°C for 10 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

Coming to result analysis of Fig. 4.3.30, the formulated sample conserves the peaks corresponding to β-turn at 1687 cm^{-1} and 1676 cm^{-1}. α-helix at 1655 cm^{-1} and random coil at 1647 cm^{-1} . β-sheet at 1639 cm^{-1} & 1628 cm^{-1} like that of native protein where as the denatured one shows additional peaks corresponding to β-sheet at 1694 cm^{-1}, 1644 cm^{-1} and1632 cm^{-1} and β-turn at 1681 cm^{-1} and 1668 cm^{-1} . Thus excipient confers protection

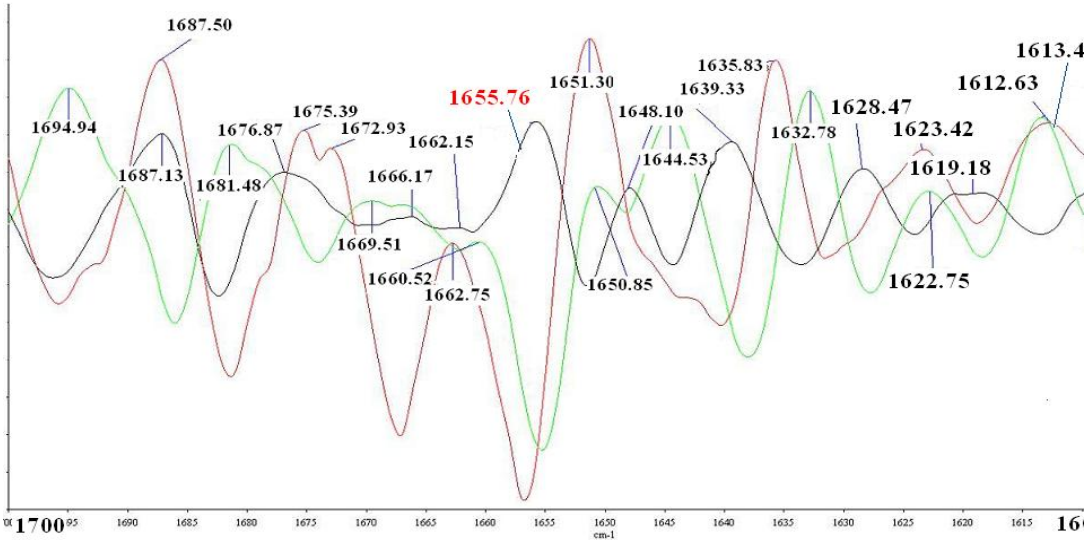

Fig. 4.3.31 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 75°C for 40 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

Analyzing the result for Fig-4.3.31, we observed that the plots of thermal denatured native protein goes side by side with that of the formulated protein, whereas control shows different peaks than that of the other two. In the formulated protein the basic peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure.

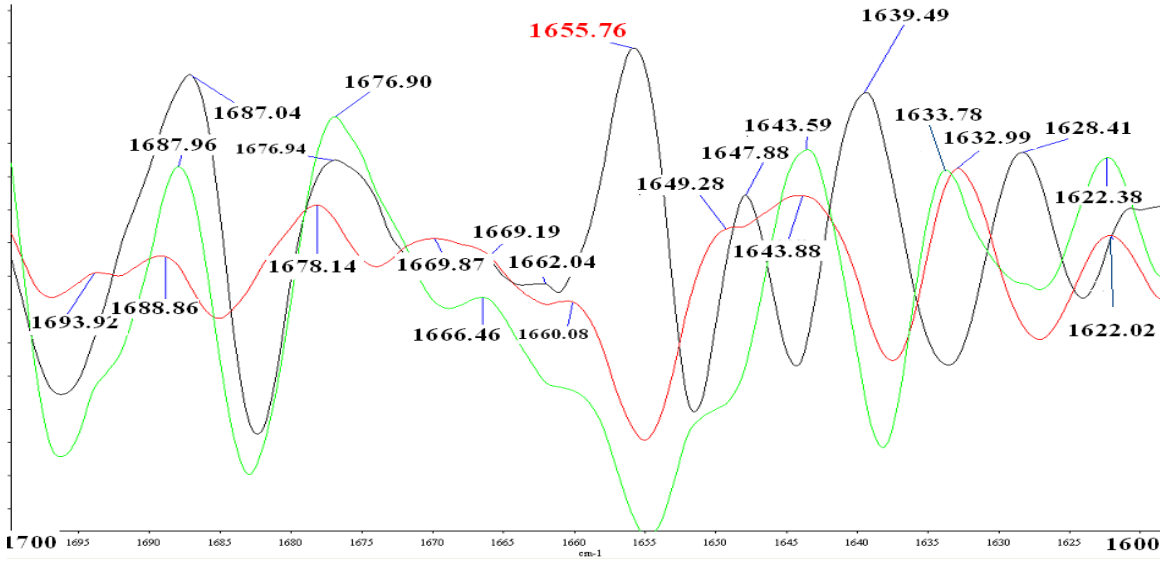

Fig. 4.3.32 FTIR plots of native protein (black line i.e. reference at room temp.) protein without excipient heat treated (red line) and formulated sample (green line- spray dried with trehalose at 75°C for 60 min treatment). The graph shows second derivative form of the original spectrum of Amide-I region (X-axis: wave number 1700-1600 cm^{-1} and Y-axis: absorbance)

In the above figure we got a peculiar result. For the higher wave numbers all the three plots of formulated sample i.e. of control, native and formulated samples, have similar kind of peaks at higher wave numbers. Whereas for lower wave number plots for each of the sample showed unique peaks. And the very basic for stability of formulated protein is the peak that corresponds to the α-helix at 1655 cm^{-1} is not conserved. So the excipient is not working in the specified temperature for the specific time of exposure.

After going through FTIR result analysis of trehalose formulated sample for each individual time interval of mentioned temperature treatment, we concluded that, for both the temperature range trehalose is not a protectant for more time of exposure to heat i.e. upto 40 minutes and 60 minutes.For less than 20 mins exposure it protects α-helix at 1655 cm^{-1} and β-sheets at 1639 cm^{-1} and 1628 cm^{-1} where denature one shows an additional β-sheet at 1644 cm^{-1} and 1632 cm^{-1} and β turn at 1681 cm^{-1} and 1668 cm^{-1}. Thus trehalose can be used as an excipient for low time of exposure to heat that is at 65°C and 75°C up to 10 minutes. But it

can't be used as an excipient at the specified temperature for time of exposure more than 10 minutes.

Finally we analyzed the complete FTIR result of each and every individual excipient i.e. ammonium sulfate, mannitol, maltodextrin, and trehalose formulated samples. We gave thermal treatment to the formulated samples at two specific temperature i.e. 65°C and 75°C for time intervals of 5, 10, 40 and 60 minutes. And going by our one of the objective, that is to choose the best excipient out of the four we used, we concluded that,

- Mannitol is the best excipient showing maximum alterations in the populations of the α-helix, β-sheet, and random coil structures and protecting upto 1hr time of exposure.
- Then comes ammonium sulfate which fails to protect time of exposure more than 40 mins at 75°C temperature treatment.
- Trehalose can't protect for longer time of exposure but works well for shorter time of exposure.
- Maltodextrin cannot be used as an excipient at all.

4.4 Detection of denaturation of protein through Calorimetry

Exposure to heat leads to deformation of bonds resulting in the physical change of the sample called phase transitions. The points at which phase transition occurs are called transition points. The physical changes involve glass transition (T_g), crystallization (T_c) and melting (T_m). Such events will either release energy (Exothermic) or it will be taken up by the system (Endothermic) and this difference can give rise to a change in the Enthalpy (ΔH_m). To measure the amount of heat absorbed or released during such transitions we used the Differential Scanning Calorimetric Technique (DSC). The result of a DSC experiment is a curve of heat flux versus temperature or versus time. Using this technique it is possible to observe fusion and crystallization events as well as glass transition temperatures (T_g). Glass transition temperature is an intermediate temperature between solid and liquid states. It has been also reported that formation of intracellular glass will increase the stability of the anhydrobiotic organism in the dry state (Liao et al. 2002). Molecular mobility also creates an adverse effect on the storage stability. Substance stored above T_g has been reported of high molecular mobility while those stored below the T_g shown to have very low molecular mobilty. T_g value is highly affected by the moisture content. Presence of moisture reduces the glass transition temperature there by changes in the physical state of the protein occurs. In

this study, glass transition behaviors of the native and formulated proteins are considered as the subject.

Sample	**T_g value**	**DeltaC_p J/(g*K)**
Control	64.8 °C	1.823
BSA + d-Mannitol	75.1 °C	1.81
BSA+ Maltodextrin	62.6 °C	3.137
BSA + Trehalose	65.5 °C	5.149
BSA + Ammonium Sulfate	64.2 °C	6.707

Table- 4.2: T_g value and change in specific heat of native and formulated samples at room temperature.

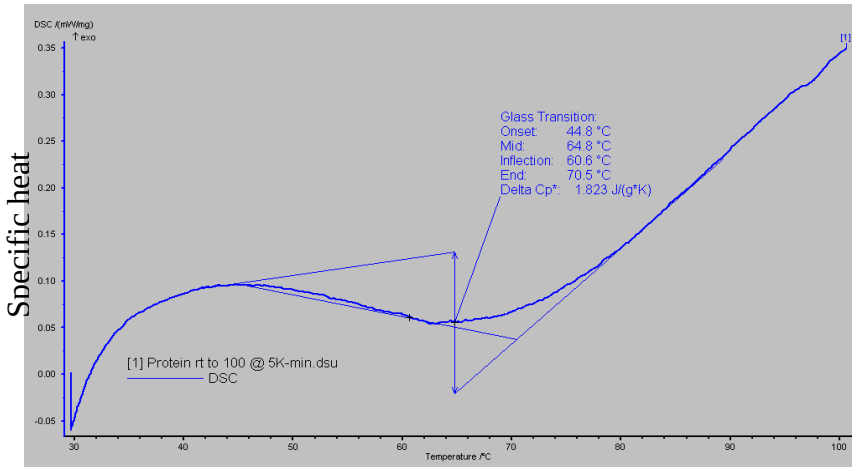

Fig. 4.4.1 T_g value of native BSA, X-axis shows temperature and Y-axis shows specific heat (cp) scan rate 5 °C per minute, temperature range from 30 °C to 100 °C. Formation of glass starts from 44.8 °C and ends in 70.5 °C and T_g can be obtained by taking the mid point i.e. 64.8°C.

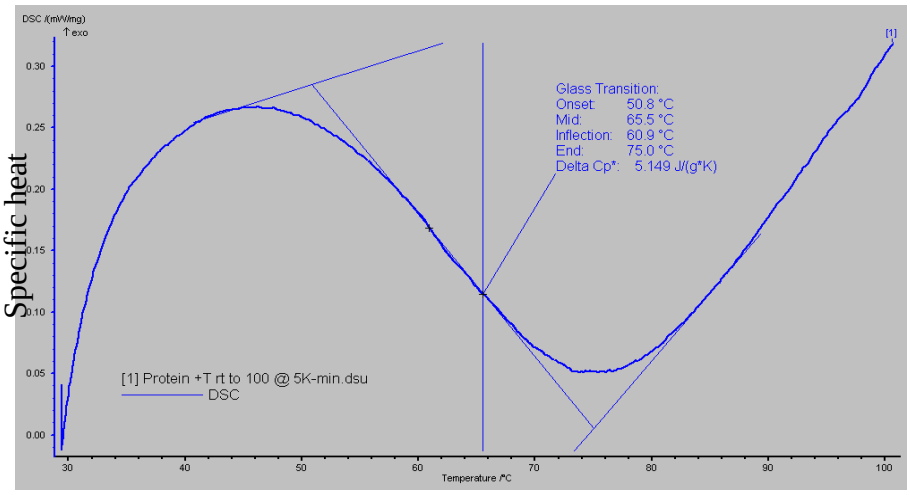

Fig. 4.4.2.T_g value of native BSA formulated with trehalose, X-axis shows temperature and Y-axis shows specific heat (cp) scan rate 5°C per minute, temperature range from 30°C to 100°C. Formation of glass starts from 50.8°C and ends in 75.0°C and T_g can be obtained by taking the midpoint i.e. 65.5°C.

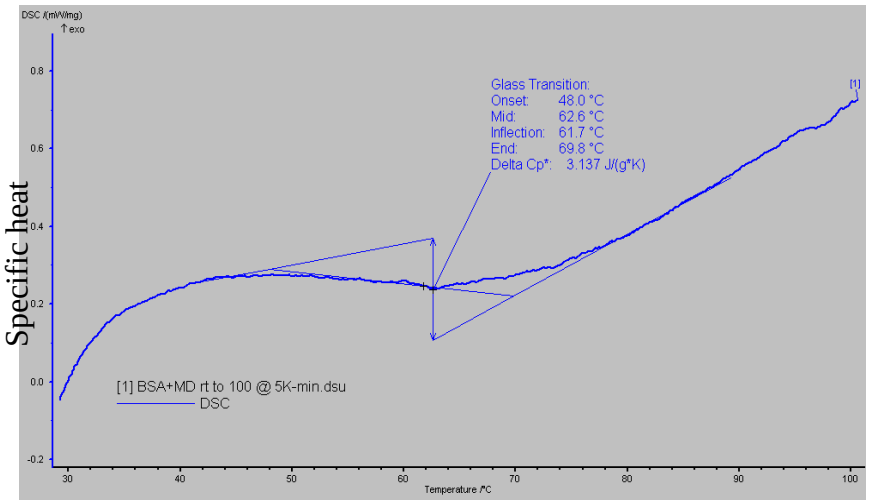

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Fig. 4.4.3. T_g value of native BSA spray dried with maltodextrin, X-axis-temperature and Y-axis- specific heat (cp) scan rate 5°C per minute, temperature range from 30°C to 100°C. Formation of glass starts from 48.0°C and ends in 69.8°C and T_g can be obtained by taking the midpoint i.e. 62.8°C.

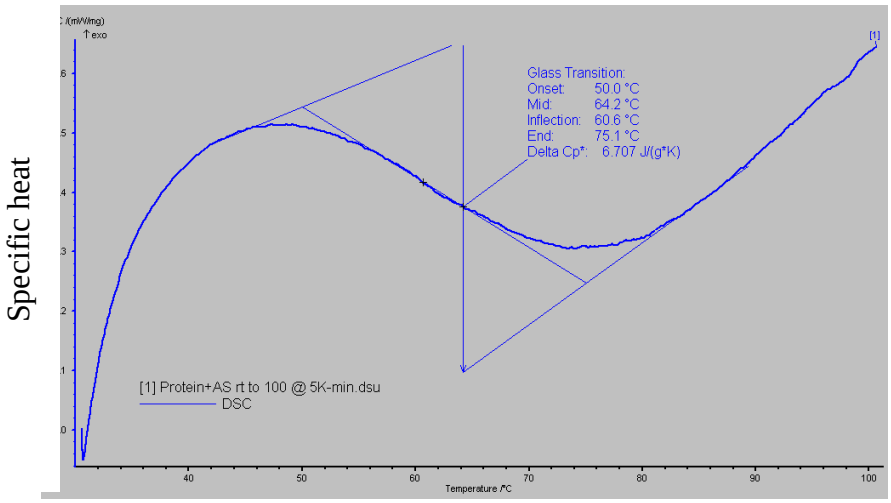

Fig. 4.4.4. T_g value of native BSA spray dried with ammonium sulfate, X-axis-temperature and Y-axis - specific heat (cp) scan rate 5°C per minute, temperature range from 30°C to 100°C. Formation of glass starts from 50.0°C and ends in 75.1°C and T_g can be obtained by taking the midpoint i.e. 64.2°C.

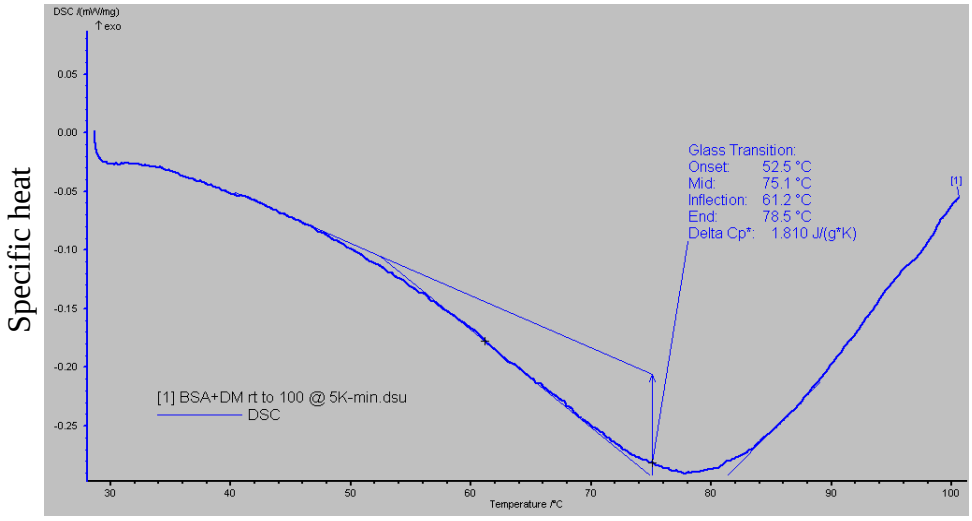

Fig. 4.4.5. T_g value of native BSA spray dried with mannitol, X-axis-temperature and Y-axis-specific heat (cp) scan rate 5°C per minute, temperature range from 30°C to 100°C. Formation of glass starts from 52.5°C and ends in 78.5°C and T_g can be obtained by taking the midpoint i.e. 75.1°C.

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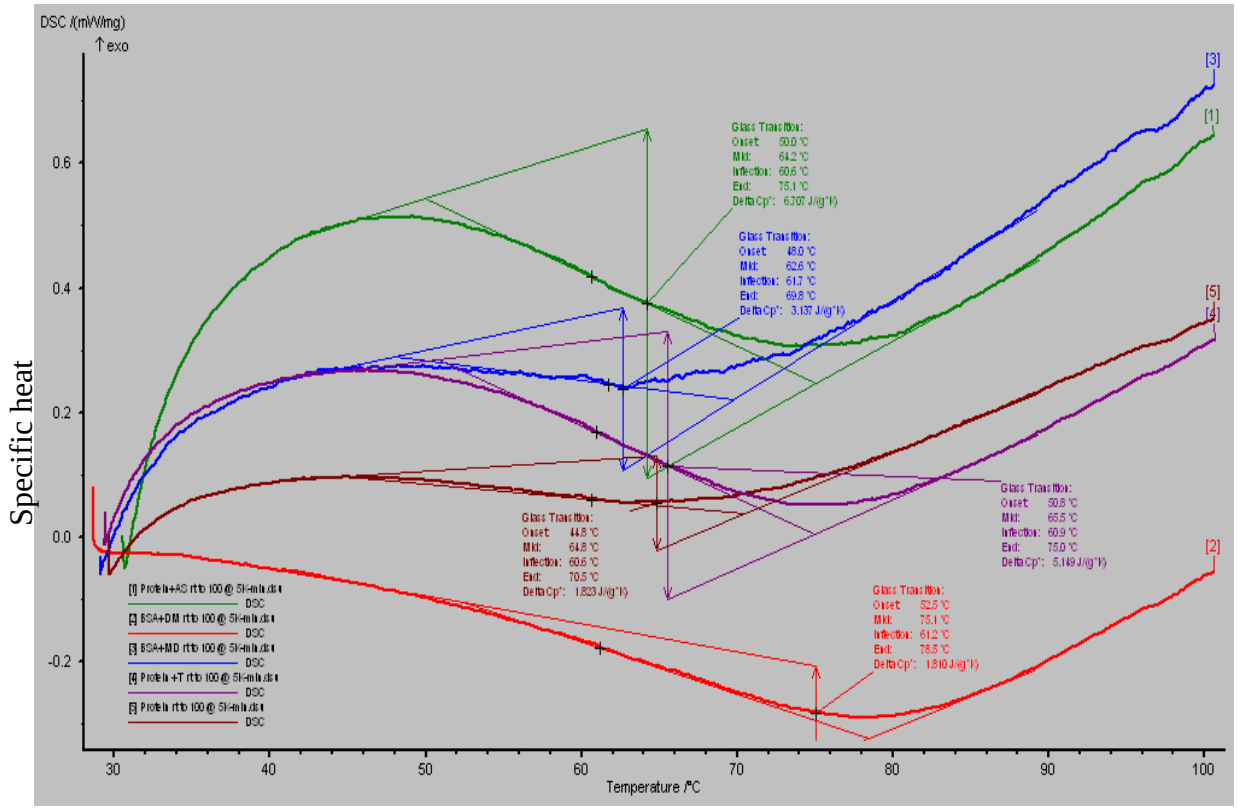

Temperature

Fig 4.4.6: Overlapping all the graphs of T_g values obtained by DSC for comparison.

From the Fig 4.4.6: it is observed that the protein formulated with d-mannitol shows the highest T_g value. This indicates that the excipient provides a better preserving condition for the protein because as the T_g of the sample increases this decreases the possibility of the phase change of the sample at room temperature and thus the physical stability of the sample increases.

Chapter 5

Summary and Conclusions

The effect of excipients like: mannitol, ammonium sulfate, trehalose and maltodextrin on the stability of the BSA were studied.

Considering the results obtained from different characterization techniques for mannitol formulated samples, it works for the protein precisely.

TGA data indicate that out of all four excipients, mannitol formulations has less moisture content i.e. 3.149%. The effect of mannitol on the decrease in the moisture content was partially attributed to the increased hydrogen bonding that occurs between mannitol and protein. As comparing to moisture content of native protein i.e. calculated as 10.354%., mannitol formulations brings a significant difference hence regarded as a good excipient (Town et al in 2000).

It preserves the helical structure of the protein thoroughly, does not allow the protein to produce any type of shift in the peak position and to form any new β-sheets or any type of additional peak hence regarded as a good excipient (Murayama et al. 2004). It preserves other peaks intact position like that of native protein even after high temperature treatment for prolong time i.e. 75°C for 1 hour heat treatment detected by spectroscopy.

From literature, it is reported that a good excipient should have capacity to increase the glass transition values in order to reduce the molecular mobility and structural alterations (Sun et al.1998). Differential scanning calorimetry detects that the mannitol formulated samples showed highest glass transition value i.e. 75.1°C there by it reduces the molecular mobility and preventing the conformational transitions and from many deleterious reactions.

Aggregation occurs due to heat was detected by electrophoresis. Mannitol gives protection to the protein from degradation even at 75°C for 60 minute (Arakawa et al. 1999).

The second excipient used was ammonium sulfate. It produces less percentages of moisture i.e. 3.607% and T_g value it is showing 64.2°C. T_g value calculated is nearly equal to that of native protein. From IR spectroscopic analysis, it was found that this excipient protects the

protein but up to a certain level. It can not give the protection at 65°C and 75°C for long times i.e. for 60 minutes. From gel electrophoresis, it is also found that ammonium sulfate also gives protection the proteins from aggregation but not up to long time of expose. Hence it is giving protection up to certain extent.

Trehalose is used as an excipient in the experiment. Trehalose is regarded as an efficient stabilizer in various studies (Yoshii et al. 2007) but in case of this protein, trehalose is not showing any significant protection. It produces hygroscopic and sticky products. The moisture content is very high i.e. 8.094%. The glass transition temperature produced by trehalose formulated samples i.e. 65.5 °C was not so high. This formulation also up to certain extent protects the helical structure of the protein but after 10 minute heat treatment at 65°C and 75°C it neither protects the helical structure of the protein nor it conserved the bands.

Maltodextrin can not be regarded as a good excipient for this protein. The formulated samples containing maltodextrin neither increases the glass transition temperature i.e. it is showing 62.8°C nor produces moisture free products i.e. 7.709%. It does not show any protection towards the helical structure that were lost due to temperature. It does not produce any effect on aggregation process. In the presence of maltodextrin, BSA also aggregates due to heat. Its presence or absence does not bring any change in the structure.

It was concluded from the result that BSA formulated with excipients can be stabilized by spray drying method. In between all four different excipients mannitol is regarded as the most excellent by fulfilling all desired parameters for BSA detected by all four analytical technique. This finding paves the path for protein stabilization through excipient mediated spray drying technique.

Future studies

It was concluded from the result that BSA formulated with excipients can be stabilized by spray drying method. It can be applied to any other proteins, enzymes and cells. The combination of mannitol and ammonium sulfate can be produced and its effect on the stability of the protein can be checked. Different ratios of combination of excipient with the protein can be tried. Thermal denaturation studies can be done at different temperature and different time point in order to know the excipient effects on the protein. Some more techniques can be applied to find out more accuracy.

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Bio-data

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